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PROVISIONAL SPECIFICATION

Applicant(s): THE UNIVERSITY OF MELBOURNE

Invention Title: PROTEASE-ACTIVATED RECEPTORS

The invention is described in the following statement:

PROTEASE-ACTIVATED RECEPTORS

This invention relates to receptors located in the epithelium and which upon activation regulate the contractility of muscle cells. In particular, it relates to protease activated receptors (PAR) such as PAR-1 and PAR-2. The invention also relates to methods of preventing and treating diseases of the airway, particularly conditions such as asthma, and diseases of vascular tissues, and also to methods of diagnosis and to screening of putative agents for prevention and treatment of such diseases.

BACKGROUND OF THE INVENTION

Protease-Activated Receptors (PAR)

Many receptors for biologically-active effector molecules are large proteins embedded in biological membranes. They serve as transducers of information encoded in effectors such as hormones and cytokines, and are also important in the mechanism of action of pharmaceutical agents. For example, receptors located within the outer regions of the cellular membrane act to transduce such information into the cell, which may then respond in a number of different ways via specific second messenger systems. Therefore, these types of receptors have specific extracellular and intracellular domains which allow information, such as hormonal signals, to be appropriately detected and processed by cells.

Protease-activated receptors (PARs) are a relatively new subtype of a superfamily of membrane receptors which have seven membrane-spanning regions, and are coupled to intracellular second messenger mechanisms via G proteins. The three known members, respectively designated PAR-1, PAR-2 and PAR-3, have been cloned, and shown to be expressed in vascularised tissues comprising endothelial and smooth muscle cells (PAR-1 and PAR-2) and platelets (PAR-1 and PAR-3). A fourth receptor, designated

PAR-4, has also recently been demonstrated on platelets of PAR-3 deficient mice, and has been cloned (Kahn *et al*, 1998); the human homologoue has also been cloned (Xu *et al*, 1998).

PARs are activated in a unique manner, which is illustrated in Figure 1. As their names indicate, limited proteolysis by specific proteases (proteinases) removes part of the extracellular N-terminal region of the receptor, so that the newly-shortened N-terminal acts as a ligand for an as yet undefined binding region on the remainder of the receptor in order to signal the cell to respond. Thus, PARs have their own inbuilt or "tethered" ligands, and the specific protease activity reveals that these latent, intrinsic ligands act as ligands in their own right rather than as exogenous effectors.

PAR-1 (Vu *et al* 1991; Coughlin *et al*, 1992) and PAR-3 (Ishihara *et al*, 1996) are activated primarily by the blood-borne protease, thrombin, which is believed to be involved in thrombosis, inflammation and mitogenic growth (De Caterina & Sicari, 1993; Dennington & Berndt, 1994; Fager, 1995). For example, thrombin causes smooth muscle in the airways to proliferate, which may cause the airway to thicken and become obstructed. PAR-1 is also located on vascular endothelial cells, where, like many other receptor types, stimulation leads to release of nitric oxide (NO) and other factors which then cause the muscle in the wall of the vessels to relax (Muramatsu *et al*, 1991; Tesfamariam *et al*, 1993: Tesfamariam, 1994, Hwa *et al*, 1996; Saifeddine *et al*, 1996). Under normal circumstances, the enzymatic activity of thrombin is strongly suppressed by a number of endogenous inhibitors.

PAR-2 differs from both PAR-1 and PAR-3 receptors in that it is activated not by thrombin, but by trypsin and trypsin-like enzymes, such as mast cell-derived tryptase (Molino *et al*, 1997). Trypsin is usually confined to the upper gastrointestinal tract after its generation by activation of its pancreatic precursor, trypsinogen.

Trypsinogen is induced in vascular endothelial cells by tissue plasminogen activator (TPA) (Koshikawa *et al*, 1997). Tryptase is released in large concentrations from mast cells (Caughey, 1994), which are believed to have a central role in the pathogenic manifestations of asthma. Tryptase stimulates mucus release, and can inactivate some peptides such as vasoactive intestinal peptide (VIP) that relax airway smooth muscle in experimental animals, thus suggesting that the PARs play a role in the aetiology of airway diseases by inducing contraction of smooth muscle cells.

In addition to tryptase, tryptase-like enzymes are released by clara cells (Yasuoka et al, 1997), which are common in the epithelium lining the small bronchi of most mammals, including humans, the trachea of the mouse, and by lymphocytes which enter the inflamed airway in large numbers. Trypsin has been localised to normal airway epithelium (Koshikawa *et al*, 1997). In addition, tryptase-like enzymes are thought to be involved in a number of inflammatory responses and diseases, such as atherosclerosis (Atkinson et al., 1994; Kovanen et al., 1995) and varicosis (Yamada et al., 1996). Furthermore and importantly , as well as directly activating mast cell degranulation via IgE-antigen recognition, the antigens of some dust mites and pollens are proteases with trypsin-like activity (Caughey, 1997). Therefore, allergens that are central to and that are the causal agents of many airways diseases have the potential to directly and indirectly activate PAR-2.

PAR-1 and PAR-2, but not PAR-3 (Isihara et al., 1997) can also be activated by short synthetic peptide sequences corresponding to those of the tethered ligands. For PAR-1 this tethered ligand is SFLLRN-NH_2 (single amino acid code), which is also known as TRAP (thrombin receptor-activating peptide). The tethered ligand sequence for mouse PAR-2 is SLIGRL-NH_2, and is referred to herein as PAR-2 activating peptide (PAR-2-AP). Therefore, these

peptides can be used to mimic enzymic PAR activation and to document the effects of PAR activation.

The genes for PAR-1, PAR-2 and PAR-3 have been cloned (Vu *et al*, 1991; Nystedt *et al*, 1994; Bohm *et al*, 1996; Saifeddine *et al*, 1996 and Ishihara *et al*, 1997). PAR-2 mRNA has been shown to be highly expressed in vascularised or endothelialised tissues such as the stomach, intestine, pancreas, kidney and liver. In the gut, PAR-2 mRNA is located mainly in epithelial cells (Bohm et al., 1996). In blood vessels, functional PAR-2 has been localised nearly exclusively to endothelial cells, where, like PAR-1, it mediates endothelium-dependent vasodilatation (Hwa *et al*, 1996; Saifeddine *et al*, 1996). It has been proposed that PAR-2 acts as a trypsin sensor in the pancreas (Bohm *et al*, 1996a), and is involved in a possible cytoprotective mechanism for gut epithelia exposed to trypsin (Bohm et al., 1996b). Apart from these proposed activities, little is known of other physiological roles for these receptors.

Following activation, PARs are inactivated by rapid internalisation, which also provides the signals for rapid generation of new receptors from intracellular pools and *de novo* protein synthesis (Hoxie et al 1993; Bohm et al 1996). This provides a powerful self-replenishing system to maintain adequate tissue levels of the receptors.

Like PAR-1, PAR-2 mediates relaxation of arteries via the release of nitric oxide (NO; Moncada et al., 1991) and of endothelium-derived hyperpolarising factor (EDHF: Garland *et al*, 1995), although the EDHF-dependent mechanism for PAR-1 is different from that for PAR-2. The mechanisms of receptor recycling also regulate the way in which endothelial cells recover their ability to respond to further protease challenge, at least within two to three hours after the first challenge. For PAR-1, this recovery process involves rapid recycling of receptors (30min-150min) without the tethered ligand sequence, but no new N-terminal receptors are produced. For PAR-2, however, fully

intact new receptors are rapidly synthesised from stable mRNA, and are inserted into the plasma membrane (Bohm *et al*, 1996). We have now found that a similarly efficient and rapid replenishment of PAR-2 occurs in the airways (see Figure 11).

PAR and the Human Vasculature

Only PAR-1 has been identified in the human vasculature (Nelken, 1992), where expression was reported to be isolated to endothelial cells in atheroma-free arteries. In vessels affected by atherosclerosis, PAR-1 mRNA was found in endothelial, smooth muscle and mesenchymal-appearing cells. Studies on human endothelial cell PAR function have been limited to the measurement of calcium fluxes in transfected cell lines (Mari, 1996) and umbilical vein endothelial cells (Ngaiza, 1991; Kruse, 1995).

Airway Diseases

The incidence and prevalence of airway diseases such as asthma and bronchitis, which are characterized by airflow obstruction, inflammation and pathological changes in airway tissue are increasing globally (Barnes *et al*, 1996a). However, it is unknown why some people develop these airway diseases, while other people exposed to the same environmental factors do not. One possibility is that the airway defences of patients who develop the disease are less efficient than those of non-afflicted subjects.

Asthmatic patients suffer from episodic airflow limitation caused by bronchospasm, oedema and thickening of the airway walls. In addition, one of the hallmarks of asthma is that the bronchi are hypersensitive to specific and non-specific stimuli, causing them to contract too much and too sensitively, thereby narrowing the airways and making breathing difficult (Barnes; 1996, Barnes *et al*, 1996b). The most widely-used treatment for asthma is administration of drugs that cause the bronchial muscles to

relax and the airways to dilate, thus restoring the ability to breathe. The most commonly used drugs for this purpose are the so-called beta-2 agonists. These drugs stimulate another subtype of the seven transmembrane, G protein-coupled receptor superfamily, the beta-2 adrenoceptors, which are located on the muscle and mediate relaxation via well-defined biochemical mechanisms. While beta-2 agonists are effective in most patients, it has recently been discovered that some asthmatics respond poorly to beta-2 agonists, and may be prone to down-regulate their responses during chronic treatment, because of genetic mutations in the beta-2 adrenoceptor sequence. Additionally, concerns have been raised about the possibility that regular use of beta 2-adrenoceptor agonists may increase the risk of death from asthma.

Airway diseases like asthma and bronchitis are predicted to continue their dramatic rate of increase in developed societies, and therefore, new therapies, procedures and methods of diagnosis, and methods of screening for prophylactic or therapeutic agents are urgently needed.

We report here that activation of PAR-2, located immunohistochemically on airway epithelium, and in some cases PAR-1, caused dilatation of mouse, rat, guinea-pig and human bronchi and bronchioles. This physiologically relevant protective response in airways was mediated mainly by a cyclooxygenase product (*eg.* PGE_2) released from the epithelium. In addition, after receptor desensitisation due to internalisation and degradation, functional PAR-2 were rapidly replenished to the cell surface by protein trafficking and *de novo* synthesis. Therefore, epithelial PARs, particularly PAR-2, are ideally structured, positioned and regulated to mount anti-inflammatory responses to serine proteases in the airways, and are potential targets for the development of new therapies for inflammatory diseases like asthma and bronchitis.

We have now surprisingly found that, contrary to the view currently accepted in the art, PAR-2 in the epithelial layer has an anti-inflammatory role in the airways, and that PAR-2 epithelial and smooth muscle cells are differentially regulated.

We have shown that epithelial PARs, in particular PAR-2, initiate important autocrine and paracrine protective tissue responses in the airways which include regulation of smooth muscle contractility, inflammatory cell migration and function, neural activity and tissue remodelling, and therefore enable new therapies for airway inflammatory diseases like asthma and bronchitis.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a protease-activated receptor (PAR), which is located in the epithelium and which upon activation regulates muscle cell contractility, and which is activated by thrombin, trypsin or both. In a preferred embodiment, the invention provides a PAR-like receptor, or an "atypical" thrombin receptor which can be activated by trypsin. This receptor may be a PAR1-like receptor. Preferably, the PAR-like receptor is a substantially purified or an isolated receptor which may be obtained by chemical synthesis or by recombinant genetic engineering.

Preferably, the PAR-like receptor upon activation induces endothelium-dependent relaxation of blood vessels such as human coronary arteries, or epithelium-dependent relaxation of airways such as the bronchioles. More preferably, the PAR-like receptor is activated by the PAR-1 activating peptide SFLLRN-NH_2 (TRAP). Even more preferably, the receptor is partially activated by the mouse PAR-2 activating peptide, SLIGRL-NH_2, or by the human PAR-2 activating peptide SLIGRV-NH_2. In a particularly preferred embodiment, the PAR-like receptor has a modified exodomain comprising a trypsin-binding domain and a modified tethered ligand-binding region. In a more

particularly preferred embodiment, the ligand-binding region is specific for a ligand which exerts a different pharmacological activity to that of the binding domain.

The receptors of the invention preferably regulate the contractility of muscle cells, such as smooth muscle cells in blood vessels or airways, so that tissue responses to an abnormal physiological conditions are alleviated. Preferably, such conditions include but are not limited to inflammation, cell migration, cell proliferation, cell or tissue re-modelling, and cell or tissue damage or injury.

In a particularly preferred embodiment, activation of the receptor induces smooth muscle cell relaxation in the airway to suppress bronchoconstrictor reflexes, inflammation, cell proliferation and airway wall thickening. However, smooth muscle cell relaxation may also be induced in other epithelialised tissues such as the gastrointestinal tract, including the stomach, duodenum, small and large intestine or colon, the bile duct and the urogenital tract.

Thus, in a second aspect, the invention relates to a method of inducing smooth muscle cell relaxation, comprising the step of activating a PAR. Preferably, the PAR is PAR-1 and/or PAR-2. The method may be performed *in vitro* or *in vivo*.

The activation of the PAR may also be used in the treatment of conditions in which relaxation of smooth muscle is desired. Thus, in a third aspect, the invention relates to a method of treating a condition mediated by smooth muscle cell contraction. In a preferred embodiment, the condition to be treated is selected from the group consisting of broncho-constrictive diseases such as asthma, allergic bronchitis, rhinitis, hayfever, and pulmonary inflammatory diseases. In a particularly preferred embodiment, conditions involving inflammation of epithelialised and muscular tissues such as those described above may also be treated. The conditions or diseases

which can also be treated in accordance with the invention include, but are not limited, to those listed in Table 1.

As defects in the structure, turnover or activation of PAR are thought to play crucial roles in the conditions described above, the ability to control these receptors provides a method of prevention of such conditions or of diagnosis of such conditions. Thus, in a fourth aspect, the invention relates to a method of preventing one or more of the conditions described above and in Table 1, preferably airway diseases such as asthma, comprising the steps of activating a PAR in accordance with the invention, and monitoring airway tone in order to assess relief from bronchospasm or airway obstruction. Other parameters may also be measured, for example the production or activity of second messengers, G protein coupling, prostaglandin release or the like. Preferably the PAR is PAR-1 and/or PAR-2. In a particularly preferred embodiment, the release of PGE_2 is measured.

In a fifth aspect, the invention relates to a method of diagnosis of a condition mediated by smooth muscle contraction, comprising the step of activating a PAR as described above and measuring cellular response(s). The types of response(s) induced can be used as an indicator of pre-disposition to one or more of the conditions described above, thereby enabling diagnosis.

This method also enables the screening of putative therapeutic or prophylactic agents for one or more of these conditions. Accordingly, in a sixth aspect, the invention relates to a method of screening putative agents for the treatment or prophylaxis of a direct or indirect condition mediated by changes in smooth muscle cell contractility, comprising the step of exposing a PAR to the putative agent and measuring the ability of the agent to activate the PAR. Preferably the PAR is PAR-1, PAR-2, or the novel PAR-like receptor of the invention.

In a seventh aspect, the invention provides a pharmaceutical composition comprising an agent which

activates the PAR of the invention, together with a pharmaceutically acceptable carrier. Preferably the PAR is PAR-1, PAR-2 and/or the novel PAR-like receptor of the invention. The agent may be a nucleotide sequence, a classical low molecular weight compound or a derivative or analogue thereof. Alternatively, the active agent may be an analogue or derivative of a polypeptide or peptide which has biological activity similar to that of SFLLRN-NH_2, SLIGRL-NH_2 or SLIGKV-NH_2, (Blackhart *et al*, 1996), and which activates PAR. Peptidomimetic agents and their derivatives are to be understood to be within the scope of the invention.

The composition of the invention may be formulated and administered in numerous forms. Some examples are solutions, suspensions, solids, powders, liposomes, micronised particles to be administered orally, parenterally, intraperitoneally, intravenously or intranasally. The compositions may be in the form of liquid mixtures, tablets, capsules, suppositories, implants, coatings, inhalants, nasal sprays or the like, as indicated in Table 1. A person skilled in the art will be able to determine the most suitable type of formulation, the mode of administration, and the duration and dosage to be given, in accordance with the condition to be treated.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

In view of the role of PAR activation in smooth muscular activity, the methods of the invention may also be applied to conditions such as diseases of the vasculature and the lymphatic system, and other organs, particularly those in which smooth muscle is present. Conditions which are amenable to the methods of the invention include but are not limited to those listed in Table 1.

Table 1

Diseases Involving PAR

(A) Diseases of the lung and airways, including but not limited to:

- Alveolitis of diverse aetiologies
- Asthma
- Bronchitis
- Bronchiolitis, including bronchiolitis obliterans
- Ciliary dyskinesis
- Pulmonary fibrosis of diverse aetiologies
- Pulmonary hypertension and its sequelae
- Sarcoidosis

Proposed galenical forms: aerosols of solutions, suspensions or dry powders, including micronised preparations; nasal sprays; liposomal formulations, including cationic liposomes for gene vector transfer.

(B) Diseases of the gastrointestinal tract, including but not limited to:

- Crohn's disease
- Gastric and gastrointestinal ulceration, including ulceration triggered by NSAID therapy
- Inflammatory bowel disease
- Intestinal adhesion induced by surgery, injury or other mechanisms

- Ulcerative colitis
- Hirschsprung's disease
- Irritable Bowel Syndrome

Proposed galenical forms: oral formulations, including encapsulated, enteric-coated and sustained release matrix formulations; suppositories; enemas; implantable gels or slow release matrixes.

(C) Diseases of the eye, including but not limited to:

- Conjunctival inflammation
- Corneal neovascularisation
- Corneal ulceration
- Glaucoma

Proposed galenical forms: drops and gels, including slow release implantable matrices; additions to contact lenses as coatings or integral matrix component.

(D) Diseases of the genitourinary tract, including but not limited to:

- Ciliary dyskinesis
- Cystitis
- Disorders of the fallopian tubes, including infertility
- Incontinence
- Pelvic inflammatory disease
- Regulation of the contractility of the uterus in pregnancy
- Urethral inflammation

(E) Diseases of the auditory canal and middle ear, including but not limited to:

- Ciliary dyskinesis
- Eustachian canal obstruction

- Otitis media

Proposed galenical forms: drops and gels, including slow release implantable matrices; additions to grommets and stents as coatings or integral matrix component.

(F) Diseases of the vasculature and lymphatics, including but not limited to:

- atherosclerosis
- ischaemia
- lymphoedema
- modulation of angiogenesis
- systemic, pulmonary and portal hypertension
- re-anastomosis
- thrombosis
- vascular reperfusion injury

Proposed galenical forms: injectables; drops; gels including slow release implantable matrices; wrappings; additions to surgical devices including stents, grommets, valves, electrodes, catheters, synthetic vessels, as coatings or integral matrix component.

Although the invention will be described further by particular reference to bronchial constriction, it is to be clearly understood that the invention may be applied to various other conditions without departing from the central underlying concept.

The term "PAR" as used herein means a protease activated receptor, or a PAR-like receptor as described above.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only, to the following non-limiting Examples and to the drawings, in which:-

Figure 1 is a schematic representation of PAR-2 receptor. The black loops depict the membrane-spanning regions in a theoretical cell. The receptor is activated by trypsin (or by other trypsin-like proteases, eg tryptase) by cleavage of the arginine^{34}-serine^{35} peptide bond amino-terminally to the arginine^{34} in the extracellular N-terminal domain. The next approximately six amino acids of the new N-terminal (called the tethered ligand sequence, solid box) now 'flip' on to another, undefined region of the remaining receptor to initiate intracellular G-protein (G) coupling and signalling, shown here as "responses". The putative tethered ligand binding region ("R") of the receptor can also be directly activated by exogenous addition of a synthetic peptide identical to the tethered ligand sequence SLIGRL-NH_2 (single letter amino acid code) designating the mouse PAR-2 activating sequence. The similar but genetically distinct PAR-1, or thrombin receptor, is enzymically activated by thrombin by cleaving a arginine^{41}-serine^{42}-bond and the synthetic tethered ligand sequence, SFLLRN-NH_2 designating the human PAR-2 activating sequence.

Figure 2 is a chart recording showing changes in isometric force in a ring of mouse bronchus contracted to approximately 70% maximal force (F_{max}) with carbachol. The characteristic spontaneous fluctuations in active force fell markedly, then recovered at two points. After the second fall and recovery, extra carbachol was added to increase the level of active force above 70% F_{max}. A high, single concentration of the PAR-2 activating peptide, PAR2-AP, (SLIGRL-NH_2) then induced a large relaxation.

Figure 3 shows the effect of increasing concentrations of nifedipine (-logM) on spontaneous and contractile agonist-induced phasic contractile activity in

isolated ring segments of human large coronary artery. The figures shows four rings of coronary artery stretched twice (arrows) to 5g resting force, then contracted with cumulatively increasing concentrations of the thromboxane A_2 mimetic, U46619 (-logM).

Figure 4 is a chart recording showing changes in isometric force in a ring of mouse bronchus contracted to 40%-50% F_{max} with carbachol, and the effects of the PAR-2 activating peptide, PAR2-AP (SLIGRL-NH_2) and trypsin in the absence and presence of nifedipine (0.3μM).

Figure 5 shows the effect of removal of the epithelium on relaxation to the PAR-2 activating peptide, PAR2-AP (SLIGRL-NH_2) in rings of the guinea-pig isolated bronchus.

(A) Chart recordings of changes in isometric force in two rings contracted to 60%-70% F_{max} with carbachol (-logM) after which PAR2-AP was added(-logM).

(B) Group data from six experiments described in (A).

Responses are expressed as percentages of the contraction to carbachol and values are mean ± SEM. Positive values represent contractions.

Figure 6 is a chart recording demonstrating the obligatory role of the epithelium in mediating relaxation to the PAR2 activating peptide, PAR2-AP (SLIGRL-NH_2, -logM), in isolated spiral strip preparations of the guinea-pig bronchus. Strips were contracted to approximately 25% F_{max} with carbachol (not shown).

Figure 7 shows chart recordings depicting both the technique used to record relaxation in isolated mouse bronchial ring preparations, and the efficacy of the PAR2 activating peptide, PAR2-AP (SLIGRL-NH_2) and thrombin receptor activating peptide, TRAP (SFLLRN-NH_2) as broncho-relaxant agents. The time calibration bar represents 40 min, 12 min and 4 min during the F_{max} contraction, the 25% F_{max} contraction with carbachol and the additions of both peptides respectively.

Figure 8 depicts the sensitivity and maximum relaxation to (A) SLIGRL-NH_2 (PAR2-AP), (B) SFLLRN-NH_2 (TRAP), (C) trypsin and (D) thrombin in isolated mouse bronchial rings with epithelium, and the effect of potential inhibitors of these responses. All responses are expressed as percentage relaxation of the initial levels of active force induced by carbachol (30%-60% F_{max}). Values are mean ± SEM from 6-9 experiments and positive values represent contractions. Drugs used were L-NOARG (100μM), a nitric oxide (NO) synthase inhibitor; HbO, (oxyhaemoglobin, 20μM), a NO scavenger, and Indo (indomethacin, 3μM) or aspirin (100μM), both of which are cyclooxygenase inhibitors which prevent the synthesis of prostaglandin.

Figure 9 shows the effect of desensitisation to trypsin (post-trypsin) and thrombin (post-thrombin) on the relaxation to PAR2-AP in ring preparations of isolated mouse bronchi. All responses are expressed as percentage relaxation of the initial levels of active force induced by carbachol (30%-60% F_{max}). Values represent mean ± SEM from 5 experiments.

Figure 10 depicts the sensitivity and maximum relaxation to authentic prostaglandin E_2 (PGE_2), a stable analogue of PGE_2, PGE_2 ethanolamide and a stable analogue of prostacyclin (PGI_2), carbaprostacyclin, in ring preparations of mouse isolated bronchi. Values are mean ± SEM (n=3), and are expressed as percentage relaxation of the initial level of active force induced by carbachol (30-60% F_{max}).

Figure 11a shows the near-complete and rapid recovery of PAR2-mediated relaxation to trypsin following desensitization to trypsin (0 minutes) in ring preparations of isolated mouse bronchi. This recovery (30 minutes) was abolished by the protein trafficking inhibitor, brefeldin A (10μm). All responses are expressed as percentage relaxations of the initial levels of active force induced by carbachol (30-60% F_{max}). Values are mean ± SEM from the number of experiments (n) shown in parentheses.

Figure 11b shows results of a second experiment demonstrating that epithelial PAR-2 receptors in mouse bronchi are regulated by rapid turnover following desensitisation to trypsin. (a) Responsiveness to trypsin (0.3 U/ml) recovered to approximately 70% of control at 45 minutes from the zero recovery time (the time at which trypsin caused no response after the desensitising concentration of trypsin (0.3 U/ml) was washed from the bath; see Methods). Time control responses to trypsin at 15, 45, 80 and 120 minute recovery were not significantly different from the initial control (not shown). (b) The recovery of trypsin sensitivity at 45 minutes was abolished by the protein trafficking inhibitor, brefeldin A (10 μM) and the translation inhibitor cycloheximide (70 μM). Both compounds had no effect on time control responses to trypsin. Values are mean ± s.e. mean from 3-12 experiments (shown in parentheses). * $P<0.01$)

Figure 12 is a schematic representation of the proposed broncho-protective role of PAR-2 in the airways. The pathways denoted by the thick solid arrows (from activation of epithelial PAR-2 by tryptase-like enzymes including trypsin to release of PGE_2, and its subsequent activation of EP_2 receptors to initiate cAMP-dependent smooth muscle relaxation or other possible beneficial actions of endogenously released PGE_2) are powerfully operational in the bronchi. The broken arrows indicate local sources of tryptase and tryptase-like enzymes and their relationship to inflammation. The same protective mechanisms would be activated by thrombin-mediated stimulation of epithelial PAR-1.

Figure 13 is a light micrograph of a 15 μm cryostat section of part the wall of a mouse bronchus, stained with haemotoxylin/eosin. The darkly-stained convoluted layer is the epithelium (E) which lies just above the lightly-stained smooth muscle cells (SMC). Magnification: 40X.

Figure 14 depicts chart recordings showing the relaxation to the PAR-1 receptor-activating peptide SFLLRN-NH_2 (TRAP), but not the PAR2-activating peptide, SLIGRL-NH_2 (PAR2-AP), in an isolated strip of epithelium-containing pig tracheal smooth muscle. SFLLRN-NH_2 (TRAP) caused a slow relaxation to near maximum of that to isoprenaline, which showed a similar slow time course. The tissue was contracted to approximately 30% of its maximum contraction to acetylcholine (F_{max}) with carbachol. During the break in the trace (20 min) the tissue also recovered its 30% F_{max} level of active force spontaneously without washout.

Figure 15 depicts chart recordings showing relaxation to (A) PAR-2 activating peptide (PAR2-AP) or SLIGRL-NH_2) and (B) trypsin in two isolated ring preparations of the rat bronchi with intact epithelium. In each case, the tissue was contracted to 50%-70% of their maximum contraction (F_{max}) to acetylcholine (30μM). R_{max} represents the maximum relaxation to isoprenaline.

Figure 16 depicts chart recordings showing relaxation to the PAR1 activating peptide SFLLRN-NH_2 (TRAP) in a single preparation of the guinea-pig isolated taenia coli, which initially contracted repeatedly with histamine (HIST; 1μM) to stable, submaximal levels of active force. At the breaks in the trace, the preparation was washed thoroughly and left to recover for approximately 30 min prior to the next contraction with histamine. (A), control; (B), after treatment with propranolol (1μM) and prazosin (1μM) to block any relaxant adrenoceptors; (C), as for (B) except the NO synthase inhibitor, L-NOARG (100μM), was added as well; (D) as for (C) except the small conductance, Ca^{2+}-activated K^+ channel (SK) inhibitor, apamin (0.1μM), was added as well.

Figure 17 is a chart recording showing relaxation to the PAR2 activating peptide (PAR2-AP or SLIGRL-NH_2) and the PAR1 activating peptide SFLLRN-NH_2 (TRAP) in an isolated strip of rat gastric fundus in which the mucosa was left intact. The tissue was contracted to

approximately 50% of its maximum contraction to KC1 (50mM) with acetylcholine (Ach). Isoprenaline was added to obtain maximum relaxation.

Figure 18 is a chart recording showing the relaxation to the PAR1 activating peptide (SFLLRN-NH_2 or TRAP), (and to a smaller extent, the PAR2 activating peptide PAR2-AP or SLIGRL-NH_2) in an isolated strip of longitudinal muscle of the human distal colon. The preparation was contracted to a stable level of active force with repeated additions of substance P (SP, w = wash). The breaks in the trace represent 10-15 min. Apamin was left in contact with the preparation for more than 30 min.

Figure 19 shows chart recordings illustrating relaxation to thrombin (a) and trypsin (b) in isolated human coronary arteries. Cumulative concentration-response curves (c, d) were generated in endothelium-intact (O) and -denuded (●) artery ring segments contracted to approximately 50% of their maximum contraction (F_{max}) in response to 125 mM KCl ($KPSS_{max}$) with U46619 as depicted in (a) and (b). The degree of relaxation is expressed as the percentage reversal of the U46619 contraction and is mean ± SEM from five separate experiments (patients).

Figure 20 shows the effects of inhibitors of nitric oxide on responses to thrombin (A), trypsin (B) and bradykinin (C) in human isolated coronary artery ring segments contracted to approximately 50% of their maximum contraction in response to 125 mM KCl with U46619. Responses to each enzyme were examined in control tissues (O) and tissues treated with a combination of N^G-nitro-L-arginine (100 μM) and oxyhaemoglobin (20 μM) (●). Data are mean ± SEM from 5-7 separate experiments (patients).

Figure 21 shows the responses to PAR-1 and PAR-2 activating peptides in human isolated coronary artery ring segments contracted to approximately 50% of their maximum contraction in response to 125 mM ($KPSS_{max}$) with U46619. Cumulative concentration-response curves were generated to

the human PAR-1 activating peptide, SFLLRN-NH_2, in endothelium-intact (O, *n*=10 from 5 patients) and-denuded (, *n*=5 from 5 patients) preparations and to the human PAR-2 activating peptide, SLIGKV-NH_2, in endothelium-intact tissues (■, *n*=5 from 2 patients). Data are expressed as mean ± SEM.

Figure 22 shows digitized traces of original chart recordings showing the effect of desensitization to thrombin (a) and trypsin (b) on relaxation to the thrombin receptor peptide ligand, SFLLRN-NH_2 (TRAP), in separate rings of human coronary artery contracted to ~50% F_{max} with 3nM (a) and 4nM (b) final concnetrations of U46619. SP = substance P; ISO = isoprenaline; Throm = thrombin; Tryp = trypsin (units of both enzymes given as U/ml). The time calibration bar represents 20 min prior to the arrow. Tissues were incubated for at least 2h with maximum concentrations of (a) thrombin and (b) trypsin, and then washed prior to contraction with U46619.

Figure 23 shows the effect of thrombin desensitization on responses to the PAR-1 activating peptide in human isolated coronary artery. Ring segments contracted to approximately 50% of their maximum contraction in response to 125 mM ($KPSS_{max}$) with U46619 were either untreated (O) or desensitized to both thrombin and trypsin (●) before cumulative concentration-response curbes to the human PAR-1 activating peptide, SFLLRN-NH_2, were generated. Data are expressed as mean ± SEM (n=8, from 4 patients).

Figure 24 shows relaxation to the PAR1-activating peptide (A;TRAP) but not the PAR-2 activating peptide (B; PAR2-AP) in two 2mm long ring segments of isolated human bronchioles (approximately 500µm internal diameter). Both segments were contracted to approximately 30% F_{max} with carbachol, 0.005% BSA was added and then TRAP and PAR2-AP were added cumulatively. Only TRAP caused concentration-dependent relaxations up to a maximum of approximately 30%

that of isoprenaline plus IBMX. Breaks in the traces represent 10 min.

Figure 25 shows relaxation to (A) thrombin and (B) trypsin in two separate 2 mm long ring segments of isolated human bronchioles (approximately 500μm in internal diameter). The set-up procedure was as described in Figure 24. Both enzymes caused slow, activity-dependent relaxation of between 30 and 60% maximum relaxation to isoprenaline plus IBMX. The traces were interrupted (5-20 minute breaks) to depict rates of onset of relaxation and maximum responses.

Figure 26 shows the immunohistochemical localisation of PAR-2 in mouse bronchi, and demonstrates that PAR-2 and PAR-1 mediates epithelium-dependent relaxation in isolated rings of this tissue.

(a) Confocal photomicrograph showing PAR-2 immunofluorescence to discrete epithelial cells (arrow) as well as smooth muscle cells (m) and fibroblasts (arrow head). In some epithelial cells, the fluorescence appeared concentrated within areas of the cytoplasm. Pre-absorption with the peptide sequence used to raise the mouse PAR-2 antibody quenched the epithelial, smooth muscle and fibroblast fluorescence (not shown). The scale bar represents 10 μm.

(b) An original, digitised chart recording of changes in isometric force in a single ring of mouse left bronchus with intact epithelium. The tissue was contracted to approximately 40% F_{max} to acetylcholine (Ach; 30 μM) with cumulative, titrated concentrations of carbachol. Note the change of gain, and that the force recovered spontaneously over the 15 minute break in the trace after maximum relaxation to SLIGRL-NH_2)

(c) Removal of the epithelium with 0.1% Triton X-100 abolished relaxations to SLIGRL-NH_2 and SFLLRN-NH_2 whereas the tissue could still sensitively relax to PGE_2.

(d) Light photomicrographs of cross sections of mouse bronchi, showing that the 0.1% Triton X-100 perfusion

technique removed the vast majority of columnar epithelial cells (arrows) with no microscopic evidence of damage to the underlying smooth muscle (m). Scale bar represents 30 μm.

Figure 27 shows the mechanisms of PAR-mediated bronchodilatation.

(a) Epithelium- and

(b) cyclooxygenase-dependent relaxations of mouse isolated bronchi to the PAR-2 and PAR-1 synthetic peptide ligands SLIGRL-NH_2 and SFLLRN-NH_2 respectively.

(c) Relaxations to trypsin and thrombin in epithelium-intact preparations were similarly abolished by cyclooxygenase inhibition. Group data from similar experiments to that described in Figure 26, except that tissues were either treated with indomethacin (3 μM) and aspirin (100 μM) to block cyclooxygenase activity or a combination of the NO inhibitors L-NOARG (100μM) and oxyhemoglobin (20μM), or were untreated. All relaxations and contractions are expressed as percentages of the initial force to carbachol (40% F_{max}) regardless of treatment. Values on the graphs are mean ± s.e.mean from 5-9 experiments, except aspirin (n=3). * $P < 0.01$. Note that the NO inhibitors had no effect on the relaxations to PAR-1- and PAR-2-activating peptides.

Figure 28 shows that the PAR-1- and PAR-2-activating peptides SFLLRN-NH_2 and SLIGRL-NH_2 respectively, act at separate receptors to cause bronchial relaxation. Desensitisation to trypsin (■) but not to thrombin (▲) abolished the responses to the PAR-2 peptide, SLIGRL-NH_2 (a), whereas relaxation to the PAR-1 peptide, SFLLRN-NH_2, was markedly inhibited following desensitisation to both enzymes (b). In both cases (●) represents control responses. Values on the graphs are mean ± s.e. mean from 5-6 experiments. * $P < 0.01$

Abbreviations used herein are as follows:

Ach	acetylcholine
ATP	adenosine 5'-triphosphate
$F_{max}/KPSS_{max}$	maximum force of contraction (grams)
FITC	fluorescein isothiocyanate
HbO	oxyhaemoglobin
IBMX	isobutyl methylxanthine
Indo	indomethacin
KPSS	potassium-containing physiological salt solution
L-NOARG	N^G-Nitro-L-arginine
NO	nitric oxide
PACAP	Pitutitary adenylyl cyclase activating peptide
PAR	Protease Activated Receptor
PAR2-AP	PAR2 Activating Peptide
SK	Ca^{2+}-activated K^+ channel
T_o	optimal tissue stretch to give maximum active force
TRAP	Thrombin Receptor-Activating Peptide, SFLLRN- NH_2
VIP	vasoactive intestinal peptide

Chemicals

Acetylcholine chloride, bovine serum albumin, bradykinin triacetate, carbachol, cycloheximide, haemoglobin (bovine plasma), histamine dihydrochloride indomethacin, (-)-isoprenaline, N^G-nitro-l-arginine, substance P (acetate salt) and α-thrombin (bovine serum) were obtained from Sigma (MO, U.S.A.). Actinomycin D, apamin, aspirin, brefeldin A, carbaprostacyclin, isobutylmethyl xanthine (IBMX), prostaglandin ethanolamide, 9, 11-dideoxy-9α 11α-methanoepoxy-prostaglandin F2α (U46619), prazosin hydrochloride and nifedipine were from Sapphire Bioscience (N.S.W., Australia). Trypsin (bovine pancreas) was from Worthington Biochem (NJ, U.S.A.) and

SLIGRL-NH_2, SLIGKV-NH_2 and SFLLRN-NH_2 were obtained from Auspep (Vic, Australia).

Stock solutions of haemoglobin (1 mM) were dissolved in 0.9% NaCl and then reduced with sodium dithionite ($Na_2S_2O_4$). Excess $Na_2S_2O_4$ was removed by passing the solution through a Sephadex PD10 size exclusion column.

Stock solutions of brefeldin A (mM), carbaprostacyclin (1mM), prostaglandin E_2 (1mM), nifedipine (10 mM) and U46619 (1 mM) were in absolute ethanol, while those for indomethacin (100 mM) and N^G-nitro-l-arginine (100 mM) were in Na_2CO_3 and $NaHCO_3$, respectively. All subsequent dilutions of these drugs were in distilled water, as were solutions of all other drugs.

Example 1 Activation of PAR in Bronchi of the Guinea-Pig and Mouse.

Guinea-pigs of either sex (250-300g) were killed in initial experiments by CO_2 asphyxia, and in later experiments by a blow to the head. Where a blow to the head was used, great care was taken to ensure that the airways did not aspirate blood. Mice (Balb/c, male and female, 20-25g) were killed by cervical dislocation and exsanguination. In both cases, the left and right bronchi were exposed and carefully dissected free from surrounding connective tissue using a dissecting microscope, excised and placed in cold, physiological bicarbonate-buffered Krebs solution of the following composition (in mM): (Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2 and glucose 11 (Stork & Cocks, 1994a). This solution was continuously gassed with a mixture of 95% O_2 , 5% CO_2 to maintain pH at 7.4 and adequate pO_2 of the solution. An approximately 3mm long ring was cut from each bronchus. In order to maximize sensitivity, some guinea-pig airways were cut as bronchial spirals, which brings several segments of smooth muscle into series.

For the guinea-pig, the preparations were suspended vertically on two stainless steel wire hooks in

organ baths containing warm (37° C), gassed Krebs solution. One wire was attached to a micrometer-driven support leg, the other to a force-displacement transducer to record changes in force.

Mouse bronchus preparations were carefully mounted horizontally on fine (40μm) stainless steel wires attached to the jaws of a Mulvany-Halpern myograph.

After 60 min at 37° C, all rings were stretched to 0.5g passive force, which had been determined in preliminary experiments to be optimal for T_0, and allowed to recover from that stretch for a further 30 min. Maximum contraction (F_{max}) in each tissue was then determined with exogenously applied acetylcholine (ACh; 30μM) followed by washout. A further 30 min equilibration time was allowed before the tissues were actively contracted to between 20% and 60% of their individual F_{max} values with titrated concentrations of carbachol (10-100nM). When these contractions maintained steady plateaus, cumulative half-log concentrations or units of enzyme activity of trypsin, thrombin, SLIGRL-NH_2 (PAR2-AP), SFLLRN-NH_2 (TRAP) prostaglandin E_2 (PGE_2) and isoprenaline were added. In some cases, tissues were treated with a range of drugs prior to contraction to approximately 50% F_{max}. These included the cyclooxygenase inhibitors indomethacin and aspirin, the nitric oxide (NO) synthase inhibitor N^G-nitro-L- (L-NOARG), the NO scavenger oxyhaemoglobin (HbO), and the L-voltage-operated Ca^{2+} channel inhibitor nifedipine.

The luminal surface of some rings of bronchi were mechanically abraded with a tapered wooden stick to remove the epithelium. The integrity of the epithelium and underlying smooth muscle, as well as the effectiveness of epithelium removal, were confirmed histologically, using 15μm cryostat sections of the bronchi stained with haemotoxylin/eosin.

Whilst it was possible to surgically abrade the epithelium in the guinea pig to test the role of the cells in the PAR-2 mediated relaxation response, as described

below, many of the animals had large amounts of mucus present in the airways during dissection. Guinea-pigs are not pathogen-free, and an abnormally high amount of mucus can be a sign of airway infection. In view of the insight that PAR-2 might be an intrinsic protective mechanism that might be compromised during airway infection, experiments were therefore designed using specific pathogen-free Balb/c mice. If PAR-2 was shown to mediate bronchorelaxation responses in this species, it would then be possible to test whether PAR-2 and PAR-1 were involved in the pathogenesis of asthma.

When bronchial smooth muscle relaxation or contraction mediated by PAR-2 and PAR-1 in the mouse was investigated, it was found that most preparations developed spontaneous, phasic contractions to carbachol, a cholinergic agonist similar to methacholine, which were superimposed on the tonic 20%-60% Fmax responses. These contractions were rhythmical, and often of large amplitude. Furthermore, they were maintained for variable times before suddenly returning to near-basal levels of active force, as shown in Figure 2. This instability, combined with the spontaneous activity, resulted in difficulty in assessing relaxations. It was also difficult to place the contraction to carbachol at a predetermined percentage of F_{max}, since it tended to be all-or-nothing until the near-maximum of the curve was reached. However, with maximum concentrations of the relaxing agents, and with appropriate time controls, fast onset and rapid near-maximum relaxations to PAR2-AP were routinely obtained, as shown in Figure 4.

Effect of Nifedipine

The effect of the L-type voltage-operated Ca^{2+} channel inhibitor, nifedipine, on the bioassay system for bronchodilators in the mouse was also examined. Results for the human coronary arteries are shown in Figure 3. Nifedipine (10nM; see panel D) blocks both the spontaneous

contractions and those which develop in response to U46619. Such treatment allows more accurate and valid measurement of relaxations at pre-set levels of now stable active force (Stork & Cocks, 1994a). Nifedipine (0.3μM) also abolished the phasic contractions of the bronchi to carbachol, and resulted in the maintenance of stable levels of tonic, active force at any predetermined level. These results are shown in Figure 4. Development of similar spontaneous activity to that shown in Figure 3 was observed. However, even with such activity present, relaxation in response to both PAR2-AP and trypsin appeared to have occurred since active force remained constant for the time taken to obtain the relaxation to each agonist (see "TIME CONTROL panel"). Nifedipine markedly inhibited the contraction to carbachol, so that higher concentrations were required to restore force to control levels. Under these conditions, however, phasic activity was absent and unequivocal concentration-dependent relaxations to PAR2-AP were readily demonstrated. Under these conditions, PAR2-AP routinely caused well-defined, concentration-dependent relaxation.

Effect of Denudation of the Epithelium

Whenever attempts to remove the epithelium from mouse bronchi or trachea were made, they invariably damaged the underlying smooth muscle, since all rings treated in this manner failed to contract to ACh. Therefore, studies were conducted using the guinea-pig to obtain information as to the possible role of the epithelium in mediating bronchial smooth muscle relaxation to PARs. Concentration-dependent relaxations to PAR2-AP were observed in six out of thirteen bronchial rings in which the epithelium was intact; the remaining seven tissues either gave no response or small contractions to PAR2-AP. In the same number of epithelium-denuded rings (n=6) from animals where PAR2-AP caused relaxation (n=6), PAR2-AP either caused a small contraction or no response, as seen in Figure 5. In a further experiment where spiral strips rather than rings

were used, PAR2-AP caused relaxation which was clearly concentration- and epithelium-dependent, as shown in Figure 6. The presence and absence of the epithelium was confirmed histologically. SFLLRN-NH_2 (TRAP) only caused concentration-dependent contractions, which were unaffected by removal of the epithelium.

Mediators of Epithelium-Dependent Broncho-Relaxation

Figure 7 shows how isolated mouse bronchi were set up to measure relaxation sensitively. After an initial passive stretch to 0.5g (Ti) and recovery, each ring was contracted with acetylcholine (Ach; 30μM). The contraction was taken as the tissue maximum and referred to as F_{max}. After washout (w) and recovery, the tissue was then contracted to approximately 40% F_{max} with titrated, cumulative concentrations of carbachol, resulting in a change in gain. When the contraction to carbachol reached a stable plateau, cumulative, half-log molar concentrations of PAR-AP and TRAP were added. The results demonstrate that both PAR2-AP and SFLLRN-NH_2 (TRAP) caused powerful concentration-dependent relaxations in this preparation. These responses were unaffected by the combined treatment with the NO blockers L-NOARG (100μM) and HbO (20μM), but were abolished by the cyclooxygenase inhibitors indomethacin (3μM) and aspirin (100μm), as shown in Figure 8. TRAP was less effective as a mediator of relaxation, and responses to this ligand were converted to concentration-dependent contractions by indomethacin and aspirin. This effect was partially blocked by L-NOARG and HbO, as seen in Figure 8. Under the same bioassay conditions, trypsin caused activity-dependent relaxation which, as for PAR2-AP, was also blocked by indomethacin. By contrast, thrombin caused only poor indomethacin-sensitive relaxation at high concentrations which, like TRAP, were converted to contractions by indomethacin. These results are shown in Figure 8. Continual exposure of the mouse bronchi to high, cumulatively increasing

concentrations of PAR2-AP (up to 100 μM) for 2h followed by washout had no effect on the sensitivity or maximum response to subsequent addition of PAR2-AP. All occurrences of relaxation were due to an indomethacin- and aspirin-sensitive mechanism, with no role for NO. Indomethacin and aspirin also converted the relaxation in response to SFLLRN-NH_2 (TRAP) to a contraction. Thrombin gave little or no relaxation in the absence of indomethacin, but like TRAP caused a contraction in its presence. In contrast, the response to PAR2-AP was virtually abolished after continual exposure of the tissue to a maximum concentration of trypsin, but not thrombin, as shown in Figure 9, indicating that trypsin and PAR2-AP activated the same receptor. PGE_2 caused potent and maximum relaxation of the mouse bronchi, as shown in Figure 10.

Example 2 Turnover of PAR-2

Turnover mechanisms are critical regulators for cells to maintain their responsiveness to PAR-activating enzymes. Therefore, if PARs are to be effective mediators of bronchoprotection, they should be rapidly replaced by new receptors once enzymically cleaved. We examined turnover of functional PAR-2 in the mouse bronchi since, unlike PAR-1, they were purely inhibitory.

Mouse bronchi were prepared as described in Example 1. Recovery of PAR2-mediated relaxation to trypsin following desensitization to the compound was then measured. The results, presented in Figures 11a and 11b, showed that bronchial PAR-2s were replaced very rapidly following activation with trypsin. Thus in each experiment complete recovery of maximum relaxation to trypsin occurred 30 min after an initial desensitising concentration of trypsin. This recovery was abolished by the protein trafficking inhibitor brefelden A(10μm) or the protein synthesis inhibitor cycloheximide. Our data show that PAR-2 were rapidly replaced after activation with trypsin,

since relaxation to trypsin returned to near-control levels within 45 minutes after the tissue was desensitised to trypsin. This complete and rapid recovery was abolished by the protein trafficking inhibitor, brefeldin A (10μM) and the translation inhibitor, cycloheximide (70μM; Figure 11b). Equally rapid turnover of cloned PAR-2 expressed in selected cell lines has been shown to be dependent on both *de novo* synthesis of new protein as well as trafficking of preformed receptors from intracellular pools. These data imply that new, fully intact PAR-2s are vital for normal functioning of the airways.

Example 3 PAR-Mediated Airway Relaxation Occurs in Rats and Pigs

The airways of both rats and domestic pigs also relaxed when PARs were activated, as shown in Figures 14 and 15.

Pig tracheal muscle strips of approximately 2 mm x 2 mm in size and with mucosa were prepared by dissecting away overlying cartilage. Strips were suspended in Krebs solution under 1g passive tension, and contracted to approximately 30% maximal contraction with carbachol (40 nM). The PAR1 activating peptide TRAP, but not the PAR2 activating peptide, PAR2-AP, produced slow onset, near maximal relaxation of the tissue comparable in extent to that induced by isoprenaline, as indicated in Figure 14.

Bronchi from Male Sprague-Dawley rat prepared as rings in the same way as for mouse bronchi produced results that were qualitatively similar to those observed in the mouse, as indicated in Figure 15. The PAR-1 activating peptide SFLLRN-NH_2 (TRAP) only caused a contraction, whereas thrombin caused a small relaxation.

These observations show that the bronchodilatory principle is general, and we have demonstrated this in four species, including two phylogenetically-related species (mouse and rat) and two more distantly-related species, the

guinea-pig and domestic pig. As shown in Example 6, these findings also extend to human airways.

Example 4 PAR Mediated Relaxation Occurs in Non-Airway Tissue, and Can Utilize Effector Mechanisms Different to those in the Mouse Bronchus

(A) Guinea-Pig taenia coli

2 cm strips of taenia coli with intact Auerbach's plexus, but which had been stripped of the mucosa, were suspended in Kreb's solution under 1 g passive tension, and contracted with histamine (1μM) to induce active tension.

This tissue relaxed in response to TRAP in a concentration-dependent manner. The relaxation was not suppressed by the cyclo-oxygenase inhibitor indomethacin (3μM), the nitric oxide (NO) inhibitor L-NOARG (100μM), the beta-adrenoceptor antagonists propranolol (1μM) or the alpha-adrenoceptor antagonist prazosin (1 μM), thus precluding prostaglandin, NO, and adrenergic mechanisms. However, the relaxation was inhibited by pre-treatment with the small conductance Ca^{2+} -activated potassium channel (SK) inhibitor, apamin (100 nM), as shown in Figure 16. There was no relaxation to ATP in the presence of apamin, indicating the selectivity of apamin for SK channels. These data indicate that the PAR-activated protective mechanism can couple to several response transduction systems, and is not limited by the availability of cyclo-oxygenase metabolism. The exact mediator of the apamin-sensitive relaxation in this tissue is unknown, but candidates include the neuropeptides PACAP and VIP and the purine ATP, which are thought to directly or indirectly open SK channels that mediate relaxation.

(B) Rat gastric fundus

Longitudinal strips of gastric fundus from male Sprague-Dawley rats were suspended under 1 g passive isometric tension in Krebs solution.

This tissue relaxed to both PAR-1 and PAR-2 activating peptides, as shown in Figure 17.

(C) Human distal colon

Human distal colon strips obtained at bowel resection were suspended at 1g passive isometric force and contracted with substance P (30nM) to maintain a steady level of active tension.

This tissue relaxed in an apparently apamin-insensitive manner to SFLLRN-NH_2 (TRAP) and to a lesser extent PAR2-AP. Thrombin, however, did not result in relaxation in this tissue, as depicted in Figure 18.

Example 5 PAR-Mediated Relaxation in Human Coronary Arteries

Human distal right coronary arteries (2-3 mm o.d.) were obtained from the explanted hearts of nine patients undergoing heart transplantation at the Alfred Hospital, Melbourne. Six patients were diagnosed with end-stage dilated cardiomyopathy, two with congenital septal defects and one with ischaemic heart disease.

Arteries were isolated immediately after explantation and transported to the laboratory in ice-cold Krebs solution (composition (mM): Na^+ 144, Cl^- 128.7, HCO_3^- 25, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2 and glucose 11). 3 mm ring segments, some with the endothelium removed by abrasion of the luminal surface with a filter paper taper moistened with Krebs solution, were mounted between two parallel, stainless steel wire hooks in 30 mL organ baths containing Krebs solution maintained at 37°C and continuously bubbled with 95% O_2, 5% CO_2. One hook was attached to a micrometer-adjustable support leg and the other to an isometric force transducer (Grass Instruments, model FT03C) to record changes in isometric, circumferential force which were amplified and displayed on flat bed chart recorders (W & W Scientific Instruments).

Following a 60 min equilibration period, passive force (5g) was applied to the artery rings, which were then allowed to recover for 30 min before again being stretched to 5 g. After a further 30 min, rings were exposed to 125 mM KCl (isotonic) Krebs solution (KPSS; (Drummond & Cocks, 1996)) to obtain a maximum contraction for each artery ring ($KPSS_{max}$). The KPSS was then replaced with normal Krebs solution and the tissues allowed to return to their optimal passive force level over 30-60 min. Nifedipine (0.3 μM) and indomethacin (3 μM) were added to inhibit spontaneous contractile activity (Stork & Cocks, 1994a) and prostanoid release, respectively.

(A) Responses to PAR activators

Aortic ring segments were contracted to approximately 50% $KPSS_{max}$ with titrated concentrations of the thromboxane A_2 mimetic, U46619 (1 to 10 nM). Once the U46619-induced contraction had reached a stable level, cumulative concentration-response curves to thrombin and trypsin (0.0001 to 1 U/ml), or the human PAR-1 activating peptide (SFLLRN-NH_2), the human PAR-2 activating peptide (SLIGKV-NH_2) or the mouse PAR-2 activating peptide (SLIGRL-NH_2) (0.01 to 100 μM) were generated in the presence of bovine serum albumin (BSA; 0.005%). At the completion of each curve, maximum endothelium-dependent and -independent relaxation for each ring segment was determined with the addition of substance P (3 nM) and isoprenaline (1 μM), respectively.

(B) Effect of nitric oxide inhibitors

To examine the contribution of nitric oxide (NO) to PAR-mediated relaxation, aortic ring segments were either left untreated or were treated with the endothelial NO synthase inhibitor, L-NOARG; (100 μM), the NO scavenger, HbO (20 μM), or a combination of these agents, before the U46619-induced contraction.

(C) Desensitization experiments

Tissues were either left untreated or were treated with cumulative additions of one of thrombin (0.1 U/ml) or trypsin (0.1 U/ml) every 30 min for 2 h in the presence of BSA (0.005%). Tissues were then washed thoroughly with Krebs solution and contracted to approximately 50% $KPSS_{max}$ with U46619. Tissues were then exposed to the enzyme (0.1 U/ml) with which they had previously been treated until no further relaxation was observed. Importantly, the tissues were washed with Krebs solution, containing an appropriate concentration of U46619 to maintain the precontraction, between treatments with each activating enzyme. This ensured that receptor desensitisation was not masked by occupation of the receptor by the tethered ligand sequence. Once desensitisation was achieved, cross-desensitisation was investigated by addition of the enzyme (0.1 U/ml) not used in the desensitisation process. Following this, cumulative concentration-response curves to the mouse PAR-1 activating peptide, SLIGRL-NH_2, were generated. Again, substance P (3nM) and isoprenaline (1μM) were then added to determine maximal endothelium-dependent and -independent relaxations, respectively.

(D) PAR-Mediated Responses

Thrombin (0.001 to 0.1 U/ml) and trypsin (0.01 to 1 U/ml) each caused rapid, enzyme activity-dependent relaxations of U46619-contracted human coronary artery rings, which were abolished upon removal of the endothelium, as shown in Figure 19. Sensitivity (pEC_{50}, -log U/ml) and maximum (R_{max}, % contraction reversal) values for thrombin were 2.5 ± 0.2 and 88.9 ± 4.9%, respectively (n=5, from five patients). Relaxations to trypsin had a similar maximum (88.1 ± 2.9%) to that for thrombin, but a significantly decreased (P<0.05) sensitivity (pEC_{50} 1.7 ± 0.1) (n=5, from five patients).

The endothelial NO synthase inhibitor, L-NOARG (100 μM), in combination with the NO scavenger, HbO (20 μM), significantly decreased both the sensitivity and maximum relaxation ($P<0.05$) of thrombin (pEC_{50} 1.0 ± 0.4, R_{max} 14.2 ± 7.1%; n=5, from five patients) and trypsin (pEC_{50} 1.3 ± 0.2, R_{max} 17.2 ± 10.7%; n=5, from five patients), as shown in Figures 20A and 20B respectively. For both enzymes, the effect of L-NOARG in combination with HbO was not significantly different from that of either HbO or L-NOARG alone. The effect of these NO inhibitors on PAR-mediated responses was also not significantly different to their effect on bradykinin (n=7, from seven patients) as shown in Figure 20C. Thus, as with other endothelium-dependent dilators of human coronary arteries, PAR-mediated relaxations appear to be mediated predominantly by endothelial cell-derived NO.

The PAR-1 activating peptide, SFLLRN-NH_2, also caused potent relaxation of precontracted human coronary artery segments, with pEC_{50} (-log M) and R_{max} values of 6.9 ± 0.1 and 95.2 ± 1.3% (n=10, from five patients), respectively. This relaxation was abolished by endothelium denudation, as shown in Figure 21. In contrast, responses to the human PAR-2 activating peptide (SLIGKV-NH_2) were significantly less (R_{max} 39.9 ± 11.0%; n=5, from two patients). Interestingly, the mouse PAR-2 activating peptide, SLIGRL-NH_2, which has a similar sequence to the human PAR-2 activating peptide and has been shown to be equally active at PAR-2 in other preparations (Blackhart *et al*, 1996), caused no relaxation.

(E) Desensitization of PARs

Desensitisation of tissues with either thrombin or trypsin caused loss of responsiveness to maximum relaxation-inducing concentrations of both enzymes, as shown in Figures 22a and 22b, indicating that the receptor(s) involved are activated by either of these enzymes. Interestingly, under these desensitizing

conditions, the maximum response to SFLLRN-NH_2 was unaffected (R_{max}, 92.0 ± 5.0), although there was a small, but significant ($P<0.05$) decrease in sensitivity (pEC_{50} 7.0 ± 0.1 vs 6.4 ± 0.2; n=8, from 4 patients), as illustrated in Figure 23.

This study is the first to show functional evidence of the presence of PAR-like receptors in isolated human coronary arteries. While the presence of mRNA encoding the first thrombin receptor, PAR-1, has previously been reported in endothelial cells of human abdominal aorta (Nelken *et al*, 1992), others have demonstrated that the presence of protease-activated receptor mRNA does not necessarily correlate with tissue responsiveness (Saifeddine, 1996). Previous evidence for functional PAR in human endothelial cells has been limited to the measurement of calcium fluxes in umbilical vein endothelial cells (Ngaiza, 1991; Kruse, 1995). However, it is important to examine the functional responses mediated by these receptors, and the studies described herein provide evidence that activators of PAR cause powerful, endothelium-dependent relaxation of human coronary arteries *in vitro*.

As has been demonstrated in the vasculature of the rat (Saifeddine *et al*, 1996) and pig (Hwa *et al*, 1996), the present studies show that U46619-contracted human coronary artery ring preparations were induced to relax by both thrombin and trypsin. However, only the peptide fragment corresponding to the human PAR-1 tethered ligand sequence (SFLLRN-NH_2) was fully active in this preparation, while the PAR-2 tethered ligand sequence (SLIGKV-NH_2) induced only a partial reversal of the U46619-induced contraction at comparably high concentrations.

Responses to both thrombin and trypsin were entirely dependent on the presence of an intact endothelium, and were virtually abolished by a combination of L-NOARG-mediated inhibition of endothelial NO production and scavenging of residual NO by HbO, indicating that PAR-

induced relaxations were mediated by endothelium-derived NO. The degree of inhibition was similar to that observed with bradykinin in this study, and is consistent with other reports that endothelium-dependent relaxation of human coronary vessels is mediated predominantly by NO for agents including bradykinin (Kemp & Cocks, 1996) and substance P (Chester *et al*, 1990). Others have also shown that PAR-mediated vasodilatation in rat (Hollenberg *et al*, 1996; Muramatsu *et al*, 1992), pig (Tesfamarium *et al*, 1993) and dog (Tesfamarium, 1994) vessels is due to endothelial cell-derived NO.

In contrast to previous reports which showed that thrombin contracted endothelium-denuded preparations of coronary artery from dog (White, 1994; Tesfamarium, 1994) and pig (Glusa & Markwardt, 1988), neither thrombin nor trypsin induced contraction of endothelium-denuded human artery preparations in the present study. The lack of contraction to thrombin may be explained by the observation that mRNA for PAR-1 was present only in endothelial cells in normal, non-atherosclerotic arteries (Nelken *et al*, 1992). Whether thrombin or the PAR-1 activating peptide can cause contraction of endothelium-free vessels obtained from patients suffering from atheroma is of interest, since Nelken *et al*, (1992) also located PAR-1 mRNA in smooth muscle cells in affected vessels.

Our observations suggest that both enzymes mediate relaxation by PAR-1 activation. However, although trypsin can cleave and activate PAR-1, as shown in Brass *et al*, 1991 and Vu *et al*, 1991, the concentrations required (≥ 25 U/ml or 50 nM) are far in excess of those observed in the present studies on human coronary arteries to cause endothelium-dependent relaxation (0.01 - 1 U/ml or 0.02 - 2 nM). The low potency of the human PAR-2 tethered ligand sequence, SLIGKV-NH_2, and the lack of activity of the equivalent murine sequence, SLIGRL-NH_2, could initially be taken as evidence for the sole presence of PAR-1 in human coronary arteries, with the specificity of this peptide

being lost at high concentrations leading to "crossover" activation of PAR-1. The human PAR-2 tethered ligand sequence, SLIGKVD-NH_2, however, does not activate PAR-1 at concentrations up to 1 mM in human platelets (Blackhart *et al*, 1996) - far in excess of those used in this study. Furthermore, structure-activity studies have shown that PAR-1 activating peptides lacking an aromatic residue at position 2 (as is the case with SLIGKV-NH_2) are incapable of activating PAR-1 in both transfected cell lines (Nystedt *et al*, 1995) and human platelets (Scarborough *et al*, 1992; Vassallo *et al*, 1992). Therefore PAR-1, and to a lesser extent PAR-2, may exist in human coronary endothelial cells. Such a conclusion, however, is contrary to the present findings that heterologous desensitisation was induced by either thrombin or trypsin.

Such evidence suggests a single receptor type. In porcine coronary arteries, which are known to express both PAR-1 and PAR-2 (Hwa *et al.*, 1996), heterologous desensitisation was observed with high concentrations of trypsin, but only homologous desensitisation occurred with thrombin (Hwa *et al.*, 1996). Thus, while cross-desensitization and the poor sensitivity of SLIGKV-NH_2 and SLIGRL-NH_2 point to the involvement of a single receptor population, the ability of relatively low concentrations of trypsin to mediate relaxation similar to those observed with thrombin is inconsistent with the view that a 'typical' thrombin receptor is involved.

One explanation for these apparently disparate results is that human coronary artery endothelial cells possess an 'atypical' thrombin receptor capable of activation by low concentrations of trypsin. For PAR-1 and PAR-3, low concentrations of thrombin cause rapid activation by means of a receptor-specific recognition site for this enzyme, termed the hirudin-like binding domain [Liu, 1991; Vu, 1991]. This thrombin-binding region is located in the extracellular amino-terminal, immediately distal to the Arg^{41}-Ser^{42} cleavage point required for

receptor activation, and allows close alignment of the thrombin catalytic site with this peptide bond (Vu *et.al*, 1991). Therefore these receptors are capable of targetting thrombin to their specific cleavage site, ensuring efficient receptor cleavage and rapid signal transduction - prerequisites for efficient cellular responsiveness. Both the mouse and rat PAR-2s are known to lack the hirudin-like thrombin binding domain (Saifeddine *et al*, 1996), and consequently are unresponsive to thrombin. However, these receptors most likely possess a similar amino-terminal recognition site for trypsin, since, like thrombin, trypsin causes high potency and rapid responses, most likely due to targeting of the enzyme to the PAR-2 cleavage site.

The 'atypical' thrombin receptor in the human coronary artery endothelial cell appears to be sensitively activated by both thrombin and trypsin via either a common or dual enzyme binding site(s). Further support for the existence of such a receptor is provided by the observation that SLIGKV-NH_2 is capable of inducing vasodilatation despite the lack of the critical aromatic residue at position 2. Therefore, without wishing to be bound by any proposed theory, we believe that the receptor responsible for endothelium-dependent relaxation of human coronary artery is a PAR-1-like receptor, which has a modified amino-terminal exodomain comprising a trypsin binding domain and a modified tethered ligand binding region containing different pharmacophore specificities.

This study also shows that complete desensitization of responses to both thrombin and trypsin had only a small inhibitory effect on the responses of the arteries to SFLLRN-NH_2, which is contrary to earlier reports using pig coronary artery (Tesfamarian, 1994; Hwa, 1996) and rat aorta (Hollenberg *et al.*, 1996). However, differences in desensitization procedures between these previous studies and the present one might provide clues as to how PAR responsiveness is regulated following enzymic activation. In the present study, high concentrations of

both thrombin and trypsin were used for 2 to 3 hours, followed by approximately 30 minutes recovery while enzyme washout and tissue contraction occurred. This resulted in complete loss of responsiveness to both thrombin and trypsin while retaining responsiveness to SFLLRN-NH_2. With a similar protocol in the pig coronary artery, homologous desensitization with thrombin and heterologous desensitization with trypsin were observed. However, in each case, responsiveness to SFLLRN-NH_2 and SLIGKV-NH_2 was maintained. In the study of Hwa *et al* (1996), a high enzyme concentration was used over a much shorter contact time (10 to 20 minutes), and importantly, the enzyme was not washed out. The results showed a loss of responses to SFLLRN-NH_2 after homologous desensitization with thrombin, and loss of both SFLLRN-NH_2 and SLIGKV-NH_2 responses following heterologous desensitization with trypsin.

The retention and loss of responses to the tethered ligand sequences following desensitization shown in the present studies and in that of Hwa *et al* (1996) may reflect the rates of internalization and recycling of PARs following enzymic activation. Both PAR-1 and PAR-2 are rapidly internalised upon enzymic activation, stimulating the mobilisation of a pool of intact, pre-formed receptors which are rapidly (<30 minutes) inserted into the cell membrane (Bohm *et al*, 1996; Hein *et al*, 1994; Hoxie *et al*, 1993). The loss of subsequent enzyme-induced responses observed by Hwa *et al* (1996) using a rapid desensitization technique could be explained by the inability of the cell to replenish cell surface receptors from its intracellular reserve over this short period. With the prolonged desensitization technique used in this study, any reserves of intracellular receptors would probably have been depleted. Despite this, responses to SFLLRN-NH_2 were only minimally affected.

Therefore, it is proposed that, once activated, human endothelial cell PAR are internalised into early endosomes, as previously reported for human erythroleukemia

cells (Hoxie *et al.*, 1993), and are then returned to the membrane without their amino-terminal exodomain. Despite the absence of this exodomain they are able to respond to exogenously-applied tethered ligand sequences. This also indicates the presence of an endogenous activator which may act independently of receptor cleavage.

Example 6 PAR-Mediated Relaxation in Human Bronchioles

Small (~500μm) bronchioles were carefully dissected from discarded sections of human left lung which had been excised at surgery from two male lung cancer patients (49 and 63 years old, Royal Melbourne Hospital). Dissection of these bronchioles required a fine-dissecting microscope and an assistant to continually flood the preparation with cold oxygenated Krebs solution to remove air bubbles and keep the tissue viable. Bronchioles approximately 2mm in length were then mounted on 40μm diameter stainless wires in a Krebs solution-filled myograph chamber, as described for the mouse bronchus preparation above. Unlike the mouse bronchi, the human bronchiole preparation developed active force spontaneously after the initial stretch to 0.5g, then recovered partially as shown in Figures 24 and 25.

Like the mouse tissues, the human bronchioles were contracted to approximately 30-40% F_{max} with titrated cumulative concentrations of carbachol. Figures 24 and 25 show that both thrombin and trypsin caused activity-dependent relaxation that was reversed by indomethacin. In contrast, only the PAR1-activating peptide SFLLRN-NH_2 (TRAP) relaxed the bronchioles. The PAR2-activating peptide, SLIGRL-NH_2 (PAR2-AP) appeared to cause some contraction, but this was most likely baseline drift.

This finding shows that thrombin and trypsin caused relaxation, but that only TRAP, not PAR2-AP, mimicked this activity. This is remarkably similar to the pattern of activity observed for PAR-mediated endothelium-dependent relaxation in the human isolated coronary artery

in Example 5, in which it was suggested that an atypical or novel thrombin-like receptor underlies these responses.

Example 7 Cellular Localization of PAR-2 in the Airway Wall

Before testing our hypothesis that airways epithelial PAR-2 are bronchoprotective, we determined their cellular localisation within the airway wall. Using an antibody directed against the carboxyl terminal of mouse PAR-2 and confocal fluorescence miscroscopy, we found specific PAR-2 immunoreactivity localised to epithelial cells, often focally within the cytoplasm, as well as to smooth muscle cells and fibroblasts in the submucosa of the mouse bronchus. These results are shown in Figure 26.

Fresh frozen, paraformaldehyde-fixed sections (14μm) of mouse bronchus were incubated with a rabbit antiserum directed against the carboxy-terminal of mouse PAR-2 (CSVKTSY, obtained from Professor Nigel Bunnett) at a dilution of 1:500 for 48 h, washed with PBS and then incubated with a biotinylated donkey anti-rabbit antiserum (Amersham) for 2 h, washed again with PBS, and then labelled with FITC-conjugated streptavidin (Amersham), all at room temperature. After a final wash in PBS, the sections were mounted in buffered glycerol and and viewed under a Biorad MRC1000 confocal scanning laser system installed on an Olympus IMT2 microscope with a krypton/argon laser. Visualisation of FITC was achieved using a 488nm excitation filter and a 522/535 nm emission filter. Images of 768 x 612 pixels were then processed using Adobe Photoshop software.

Our demonstration that PAR-2 immunoreactivity was often localised in discrete cytoplasmic regions of airway epithelial cells supports the concept of rapid turnover from intracellular stores which was demonstrated in Example 2. Furthermore, we were unable to demonstrate specific localisation of PAR-2 mRNA in mouse bronchi using *in situ* hybridisation, whilst readily detecting PAR-2 mRNA

in the same tissue via reverse transcriptase-polymerase chain reaction (RT-PCR). This discrepancy supports the idea that there are intracellular stores of receptors which are filled by translation of stable mRNA segments of low transcript number. Therefore, the capacity of airways epithelial cells *in situ* to rapidly replenish functional PAR-2 following their enzymatic activation provides additional evidence that epithelial PARs are involved in protection of the bronchial wall during inflammation.

Example 8 PAR-Mediated Relaxation in Mouse Bronchioles

In addition to the findings in mouse bronchi, shown in Examples 1 and 2, indomethacin-sensitive relaxations to both the PAR-1- and PAR-2-activating peptides were also observed in first branches of the main bronchi of the mouse, which we have termed bronchioles. However, the sensitivity and maximum relaxation to SFLLRN-NH_2 (pEC_{50}, 5.5 ± 0.02; R_{max}, 58 ± 10%) and SLIGRL-NH_2 (pEC_{50}, 5.1 ± 0.05; R_{max}, 58 ± 4%) were significantly less ($P<0.05$) than those shown in Figure 2 for the main bronchi. Similar responses to SLIGRL-NH_2 were observed in other species. Thus, SLIGRL-NH_2 caused indomethacin-sensitive relaxations in rat bronchi (pEC_{50} 5.5 ± 0.1; R_{max} 56 ± 5%) and bronchioles (pEC_{50} 5.1 ± 0.1; R_{max} 67± 5%) and similar potency (pEC_{50} 5.4 ± 0.2), epithelium-dependent relaxation in the guinea-pig but with significantly ($P<0.05$) lower efficacy (R_{max} 31 ± 5%) than in both rat and mouse bronchi. Furthermore, in preliminary experiments we observed PAR-2-mediated relaxation in human bronchi (n=4), which in one case was blocked by indomethacin. The similar potencies for SLIGRL-NH_2 in mice, rats and guinea-pigs indicate expression of a similar receptor, whilst the different efficacies suggest either different receptor numbers or coupling between species. The rank order of efficacies for SLIGRL-NH_2, mouse > rat > guniea-pig, however, contrasts with the severity of symptoms in allergic models of asthma. For example, mice show resistance to immunological

challenge including only a small degree of airway hyperreactivity (AH) compared with rats and guinea-pigs, the latter of which show characteristic high levels of AH[16] and may die when exposed to similar immunological challenges (Ikegami *et al*, 1997). One reason why mice appear relatively asymptomatic when used in immunological models of asthma (Persson *et al*, 1997) may in part be due to a higher relative effectiveness of their PAR-2-dependent bronchoprotective mechanism.

The mouse PAR-2 tethered ligand sequence, SLIGRL-NH_2 (Nystedt *et al*, 1994) and trypsin each caused concentration-, epithelium- and cyclooxygenase-dependent, rapid onset and near-maximum relaxations of mouse bronchial rings contracted with the stable muscarinic agonist carbachol, as shown in Figures 26b and c and Figure 27. For SLIGRL-NH_2 the sensitivity (pEC_{50}, -log M) was 5.6 ± 0.1 and the maximum relaxation (R_{max}) was 94 ± 3%. Similar concentration-dependent relaxations were also obtained to the PAR-1 tethered ligand sequence SFLLRN-NH_2 (Déry *et al*, 1998) (pEC_{50}, 5.6 ± 0.1; R_{max}, 76 ± 11%) and thrombin. In contrast to PAR-2, however, both removal of the epithelium and inhibition of cyclooxygenase with either indomethacin or aspirin unmasked direct smooth muscle contractions to PAR-1 activation, as shown in Figures 26 and 27. Neither of the relaxations to SLIGRL-NH_2 and SFLLRN- NH_2 was due to nitric oxide (NO[11]), since they were completely unaffected by the NO synthase inhibitor, N^G-nitro-L-arginine (100 μM) either alone, or in combination with the NO scavenger, oxyhaemoglobin (20 μM), a combination of NO inhibitors which abolishes all NO release from vascular endothelial cells *in situ* (Drummond and Cocks, 1996; Kemp and Cocks, 1997). Therefore these results indicate that a prostanoid released from the epithelium mediated the relaxations to both PARs. PGE_2 is a likely candidate, since it is the most prevalent prostanoid released from the airway epithelium (Pavord and Tattersfield, 1995), as shown in Figure 26, and we found it to be a potent bronchodilator in

this tissue, causing 100% relaxation with a pEC_{50}, of 8.2 ± 0.1 (n=6) .

The relaxations to SLIGRL-NH_2 and SFLLRN-NH_2 in the mouse bronchi were likely to have been due to activation of separate receptors, since those to SLIGRL-NH_2 were abolished by prior desensitisation to trypsin but not thrombin whilst those to SFLLRN-NH_2 were inhibited by both thrombin and trypsin, as shown in Figure 28. Also, desensitisation with SLIGRL-NH_2 blocked the response to trypsin. This pattern of activity agrees with previous reports showing that thrombin only activates PAR-1 while higher concentrations of trypsin can activate both PAR-2 and PAR-1 (Vu *et al*, 1991; Molino *et al*, 1997).

PAR-Like Receptor

Activation of PAR-like receptor results in endothelium-dependent, NO-mediated relaxation of contracted human coronary arteries *in vitro*. This receptor either has a common, low stringency 'hirudin-like' thrombin binding domain, or other binding domains such that serine proteases other than thrombin (*eg*. trypsin) can sensitively activate it. This receptor also appears to be recycled via a mechanism whereby cleaved (activated) receptors are returned to the membrane, and are able to respond to agonists acting independently of receptor cleavage. The pathophysiological roles of endothelial cell PARs in human coronary arteries are unknown, although one possibility is that by inducing a vasodilator response, these receptors may limit the degree of thrombosis following plaque invasion by mast cells, as this process is known to be associated with release of proteases such as the trypsin-like enzyme, tryptase (Kovanan *et al*, 1995). The non-selectivity to activation of this novel PAR receptor by thrombin and trypsin may also extend to other proteases.

The studies described herein have identified functional PAR-1 and PAR-2 in the bronchi of mouse, rat,

domestic pigs and guinea-pigs which, when activated by specfic proteases, thrombin and trypsin or the human PAR-1 and mouse PAR-2 tethered ligand sequences, SFLLRN-NH_2 and SLIGRL-NH_2 respectively, cause profound relaxation of bronchial muscle. PAR-2, and most likely PAR-1 are located in the epithelium, and when activated mediate smooth muscle relaxation via the release of endogenous PG, most likely PGE_2. This relaxation was as rapid and complete as that for isoprenaline, the clinically most efficacious and rapidly acting beta-adrenoceptor agonist bronchodilator currently available.

We have demonstrated for the first time that two types of PAR receptors, PAR-1 and PAR-2, are located on bronchial epithelial cells. Without wishing to be limited by any proposed mechanism, we postulate that activation of these receptors mediates relaxation of the airway by stimulating release of prostaglandin (PG), most likely PGE_2, an endogenous local hormone. This relaxation is as efficient and rapid as that elicited by the most effective known bronchodilator drugs, the beta-2-adrenoceptor agonists, exemplified here by isoprenaline. Therefore, the findings described herein demonstrate that activation of PAR stimulates activation of a potent and highly efficient protective mechanism that operates to keep the airways open. Furthermore, the PGE_2 released by PAR activation may have an important role in protecting airway tissue from pathological change by regulating tissue responses to injury and regulating mucosal immunity.

Our studies are not only the first to describe functionality for PAR-2 and PAR-1 in the airways, but they also show that PAR-2 activation results in powerful epithelium-dependent bronchodilatation with no evidence for direct contraction, even though PAR-2 were also localised on smooth muscle cells. As described here in the mouse, PAR-2 are also expressed in both the epithelium and smooth muscle cells of human airways (D'Andrea *et al*, 1998; published after the priority date of this application).

This dual localisation of PAR-2 to the mucosal and submucosal layers of the airways is important, however, since it reconciles our proposal that epithelial PAR-2 are anti-inflammatory with the current dogma that like PAR-1, PAR-2 are pro-inflammatory (Déry *et al*, 1998), possibly being activated by mast cell-tryptase (Molino *et al*, 1997).

Thus, we propose a dual compartment model for the role of PAR-2 in the airways. In this model, anti-inflammatory epithelial PAR-2 (compartment 1) normally override any pro-inflammatory effects of smooth muscle and perhaps fibroblast PAR-2 (compartment 2) activated by mast cell tryptase, since PGE_2 potently inhibits mast cell activation (Pavord and Tattersfield, 1995). It is interesting to note that trypsin has been localised in epithelial cells of normal human airways (Kawano *et al*, 1997). We have confirmed this finding and furthermore localised specific trypsinogen immunofluorescence to Clara cells in human bronchi. Therefore, in our model, epithelial and smooth muscle PAR-2 may be differentially regulated by specific tryptic enzymes released preferentially in each compartment.

Based on these findings and the vasodilator effects of PARs in blood vessels, it appears that PAR activation is a general protective mechanism relevant, but not limited, to epithelia of bronchi and vessels, mucosal surfaces and joint connective tissues. Defects in this system may be important determinants of disease susceptibility and severity. Since PARs are activated by tissue injury and proteases are released both during innate and acquired immune responses, this invention has broad application to numerous disease states where deficient intrinsic protection from injury contributes to disease pathogenesis and/or severity. Therefore, the invention has wide application in the design of diagnostic and therapeutic strategies for managing these conditions.

The invention makes possible new treatments for many, if not most, inflammation-related diseases of the

airway. It may also apply to atherosclerosis in blood vessels, as well as to similar inflammation-mediated diseases of other muscle-lined tubes in the body, such as the bile duct, urogenital tract *etc*).

The mechanism of relaxation of the PAR of the invention offers scope for avoiding the limitations of beta-2 therapy. Treatment of inflammation-induced insufficiency in airways, for example in asthma, via this new pathway involves activation of a naturally-occurring system. Thus, it offers the prospect of being able to cure these diseases with gene therapy techniques, particularly given the easy route of access for adding the extra copies of the PAR-2 gene. It also offers a possible solution to the long-standing problem as to why the airways of healthy individuals are protected from obstruction, whereas those of asthmatics are not protected.

Furthermore, the need for vascular endothelial and airway epithelial cells to replace PAR-2 quickly implies that they serve a protective function, rather than causing cell damage as previously believed, due to the potentially deleterious effects of mast cell-derived tryptase in preventing or treating infection.

The findings described herein not only demonstrate that blood vessels and airways are similar in that their inner lining cells possess powerful smooth muscle relaxing mechanisms, but also suggest that PARs may orchestrate a more general endogenous protective tissue response to inflammatory challenge and disease, which includes regulation of smooth muscle contractility, inflammatory cell migration and function, neural activity and tissue remodeling. PARs are ideally configured for such a role. They are in effect 'caged', theoretically lying dormant until activated by specific proteases, many of which are known to be involved in airway immune and inflammatory responses, as depicted in Figure 12. Furthermore, following activation they are inactivated by rapid internalisation, which then signals equally rapid

replenishment of new receptors from intracellular pools and *de novo* protein synthesis from stably expressed mRNA.. Finally, the location of PARs to the epithelium is ideal for mediating such protease-dependent responses to airborne allergens, particularly suppression of contractility in the underlying smooth muscle, as shown in Figure 13.

Prostaglandin E2 and asthma

Although the potentially beneficial effects of PGE_2 as a relaxant agent, modulator of immune responses and regulator of tissue response to injury have been appreciated for some time, it has not proven possible to deliver PGE_2 or mimetics safely to the airways (Nizankowska *et al*, 1985; Daniell *et al*, 1994 and Melillo *et al*, 1994). The major limitation to exploiting the benefits of PGE_2 has been that exogenous PGE_2:

(i) potently activates sensory nerves in the airways, causing severe coughing (Costello *et al*, 1985; Stone *et al*, 1992), and

(ii) dysregulates airway mucosal blood flow (Laitinen *et al*, 1987).

Regardless of these limitations for exogenously applied PGE_2, PGE_2 has several actions likely to be of considerable benefit in asthma. PGE_2 suppresses cholinergic bronchoconstrictior reflexes at the level of acetylcholine release. PGE_2 potently inhibits activation of macrophages and lymphocytes, both of which are implicated in the pathogenesis of chronic human asthma. PGE_2 also suppresses the formation of new tissue matrix by inhibiting activation of mesenchymal cells such as airway fibroblasts. It is of considerable interest that asthmatics may die from catastrophic bronchospasm if cyclooxygenase is inhibited. There is also a large body of evidence that PGE_2 can be generated by the normal epithelium of airways, as well as by macrophages and airways cartilage. PGE_2 administered by aerosol protects asthmatics from exercise-induced asthma and from induced

mediator-induced bronchospasm (eg with methacholine), although it is tolerated very poorly.

Therefore, the invention represents a novel method to harness the therapeutic potential of PGE_2 by causing its endogenous release within tissues.

In conclusion, and without wishing to be bound by any proposed mechanisms for the observed advantages, it appears that PARs mediate powerful epithelium-dependent brochodilatation, most likely via PGE_2, which offers scope for new and effective therapies for airway inflammatory diseases like asthma and bronchitis. Also, individuals susceptible to inappropriate loss or down-regulation of the PAR-2 protective defence would be more likely to develop disease or diseases of increased severity; this finding provides the basis for new diagnostic and prognostic methods.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in the specification.

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Br. J. Pharmacol., 1994a 113 1093-1098.

Stork, A.P. and Cocks, T.M.
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Xu *et al*
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Surgery, 1996 119 494-497.

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Am. J. Respir. Cell Mol. Biol., 1997 16 300-308.

CLAIMS

1. A PAR-like receptor which is

a) present in human coronary artery endothelial cells;

b) able to induce relaxation of smooth muscle;

c) activated by thrombin and trypsin;

d) activated by the PAR-1 activating peptide SFLLRN-NH_2 (TRAP); and/or

e) partially activated by the mouse PAR-2 activated peptide, SLIGRL-NH_2 or the human PAR-2 activating peptide, SLIGRV-NH_2.

2. A PAR-like receptor according to Claim 1, in which the ligand binding region is specific for a ligand which exerts a different pharmacological activity to that of the binding domain.

3. A PAR-like receptor according to Claim 1 or Claim 2, which has a modified exodomain comprising a trypsin binding domain and a modified tethered ligand-binding region.

4. A PAR-like receptor according to any one of Claims 1 to 3, whose activation is mediated by endothelium-derived nitric oxide.

5. A method of inducing relaxation of smooth muscle in a subject in need of such treatment, comprising the step of activating a protease-activated receptor (PAR) in an epithelial or endothelial cell.

6. A method of prevention or treatment of a condition mediated by smooth muscle cell contraction, comprising the step of activating a protease-activated receptor (PAR) in an epithelial or endothelial cell in a subject in need of such treatment.

7. A method according to Claim 6, in which the condition involves inflammation of epithelialized and/or smooth muscular tissue.

8. A method according to Claim 6, in which the condition is a disease of the vasculature or lymphatics.

9. A method of regulating the contractility of smooth muscle cells, comprising the step of activating a protease-activated receptor (PAR) in an epithelial or endothelial cell.

10. A method according to Claim 9, in which the smooth muscle cells are present in blood vessels, airways, or epithelialized tissues, thereby to alleviate a tissue responses to an abnormal physiological condition selected from the group consisting of inflammation, cell migration, cell proliferation, cell or tissue re-modelling and cell or tissue damage or injury.

11. A method according to Claim 9 or Claim 10, in which activation of the receptor induces smooth muscle cell relaxation in the airway to suppress one or more of bronchoconstrictor reflexes, inflammation, cell proliferation and for airway wall thickening.

12. A method according to Claim 9 or Claim 10, in which smooth muscle cell relaxation is induced in the gastrointestinal tract, including the stomach, duodenum, small and large intestine or colon, the bile duct and the urogenital tract.

13. A method according to any one of Claims 1 to 12, in which the PAR is PAR-1 and/or PAR-2, or is a PAR-like receptor according to any one of Claims 1 to 4.

14. A method according to any one of Claims 1 to 13, in which the activation of the PAR induces endothelium-dependent relaxation of blood vessels.

15. A method according to any one of Claims 1 to 13, in which the activation of the PAR induces epithelium-dependent relaxation of airways.

16. A method according to Claim 15, in which the condition is a bronchoconstrictive disease.

17. A method according to any one of Claims 1 to 13, in which activation of a PAR receptor mediates smooth muscle cell relaxation by stimulating release of an endogenous prostaglandin.

18. A method according to Claim 17, in which the prostagland is PGE_2

19. A method according to any one of Claims 6 to 18, in which the condition is asthma and PAR in bronchial epithelium is activated, thereby to stimulate release of endogenous prostaglandin E_2.

20. A method according to Claim 15, in which the condition to be treated is selected from the group consisting of asthma, allergic bronchitis, rhinitis, hayfever, pulmonary inflammatory diseases, alveolitis, infectious bronchitis, bronchiolitis, ciliary dyskinesis, pulmonary fibrosis, pulmonary hypertension and its sequelae, and sarcoidosis.

21. A method according to Claim 15, in which the condition is selected from the group consisting of atherosclerosis; ischaemia; lymphoedema; modulation of angiogenesis; systemic; pulmonary or portal hypertension; re-anastomosis; thrombosis and vascular reperfusion injury.

22. A method according to Claim 15, in which the condition affects the gastrointestinal tract, the genito-urinary tract, the eye, the ear, or the bile duct.

23. A method according to Claims 15, in which the condition is selected from the group consisting of Crohn's disease, gastric and/or gastrointestinal ulceration, inflammatory bowel disease, intestinal adhesion, ulcerative colitis, Hirschsprung's disease, irritable bowel syndrome, cystitis, disorders of the fallopian tubes, incontinence, pelvic inflammatory disease, regulation of the contractility of the uterus in pregnancy, urethral inflammation, conjunctival inflammation, corneal neovascularisation, corneal ulceration, glaucoma, ciliary dyskinesis, Eustachian canal obstruction, and otitis media.

24. A composition comprising a pharmacologically active agent which activates epithelial or endothelial PAR-1, PAR-2 or a PAR according to any one of Claims 1 to 4, together with a pharmaceutically-acceptable carrier.

25. A composition according to Claim 24, in which the PAR is epithelial PAR-1 or PAR-2.

26. A composition according to Claim 24, in which the PAR is endothelial PAR-1 or PAR-2.

27. A composition according to Claim 24, in which the PAR is as defined in any one of Claims 1 to 4.

28. A composition according to any one of Claims 24 to 25, in which the active agent is an analogue or derivative of a polypeptide or peptide which has biological activity similar to that of SFLLRN-NH_2, SLIGRL-NH_2 or SLIGKV-NH_2, and which activates PAR.

29. A composition according to any one of Claims 24 to 28, in which the active agent is a peptide selected from the group consisting of SFLLRN-NH_2, SLIGRL-NH_2 and SLIGRV-NH_2 or a peptidomimetic analogue thereof which has the ability to activate PAR.

29. A method of screening putative agents for treatment or prophylaxis of a condition directly or indirectly mediated by changes in smooth muscle cell contractility, comprising the step of exposing a PAR to the putative agent, and measuring the ability of the agent to activate the PAR.

30. A method according to Claim 29, in which the PAR is epithelial or endothelial PAR-1 or PAR-2, or a PAR-like receptor according to any one of Claims 1 to 4.

PAR-2

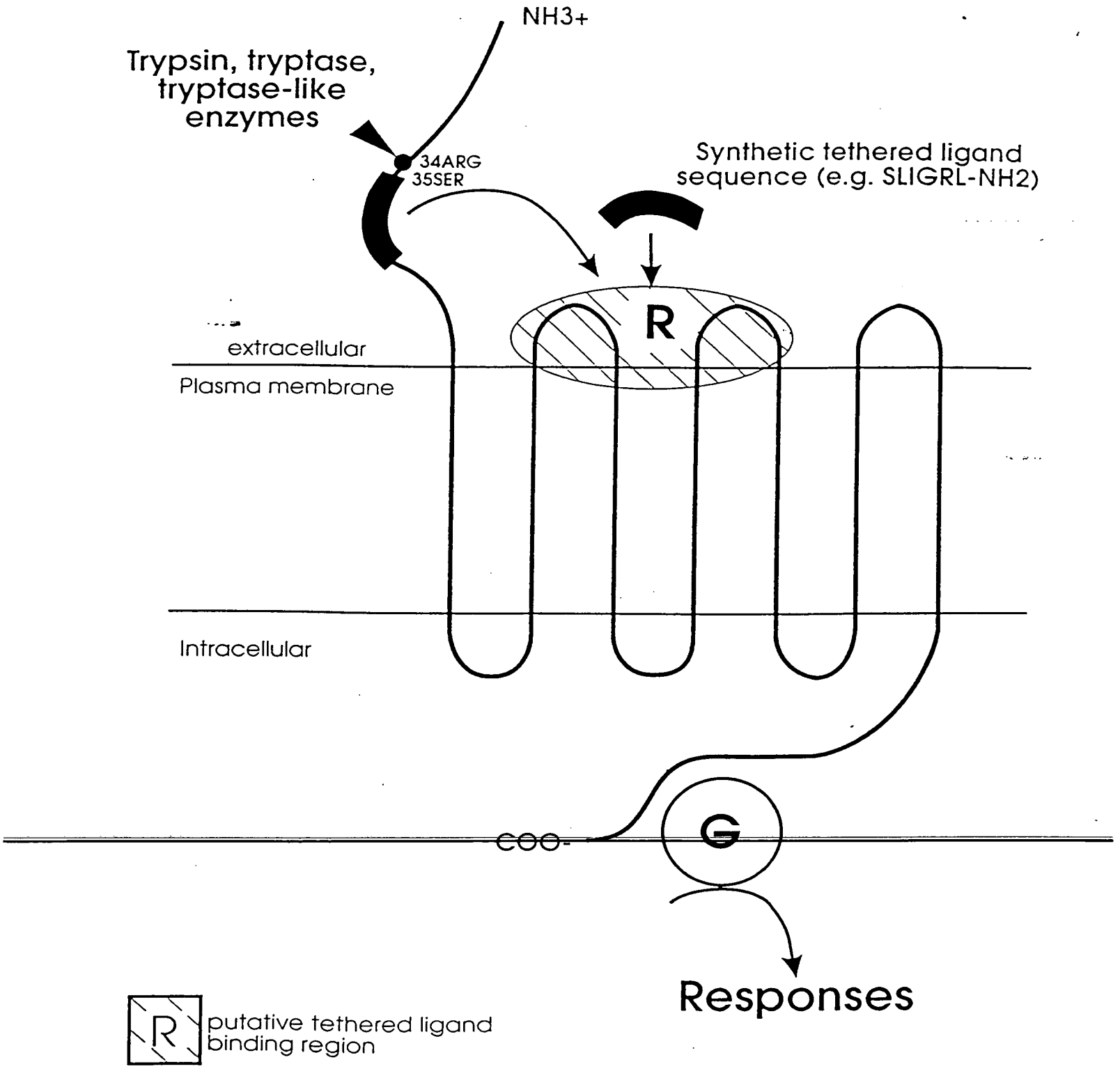


Figure 1

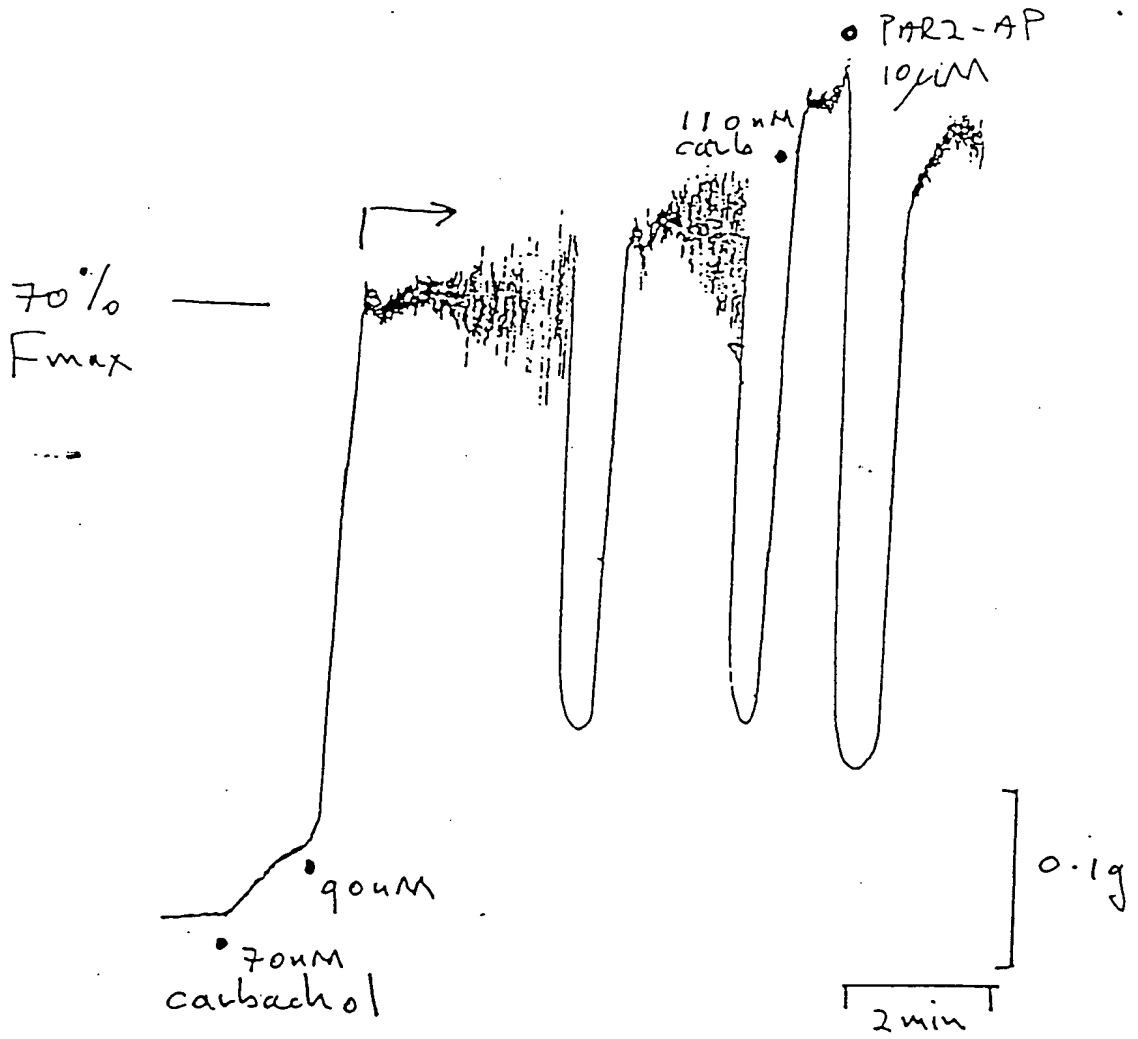


Figure 2

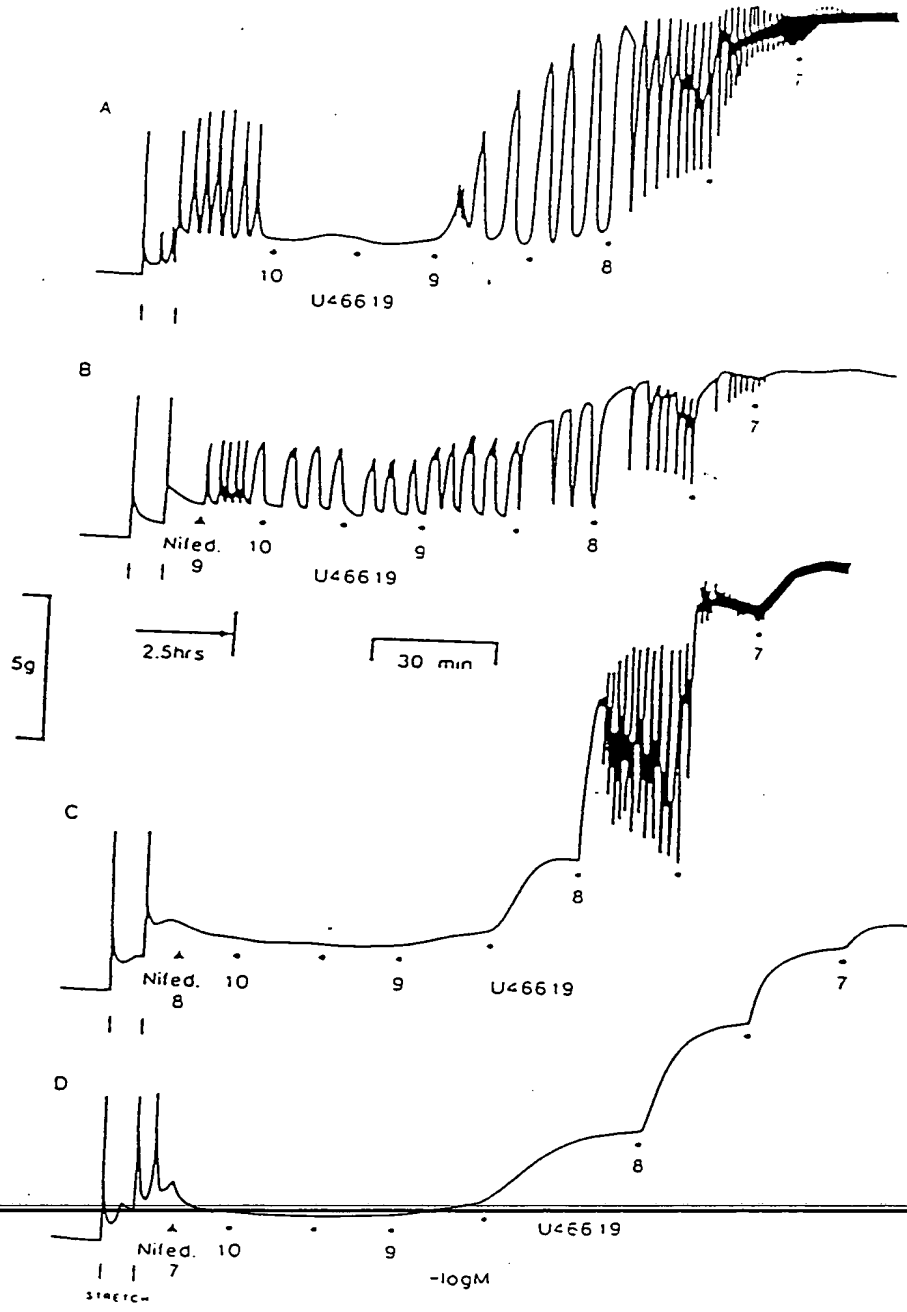

A
10
9
8
U46619
B
7
Nifed.
10
9
8
9
U46619
5g
2.5hrs
30 min
7
C
8
Nifed.
10
9
U46619
8
7
D
8
U46619
Nifed.
10
9
7
-logM

Figure 3

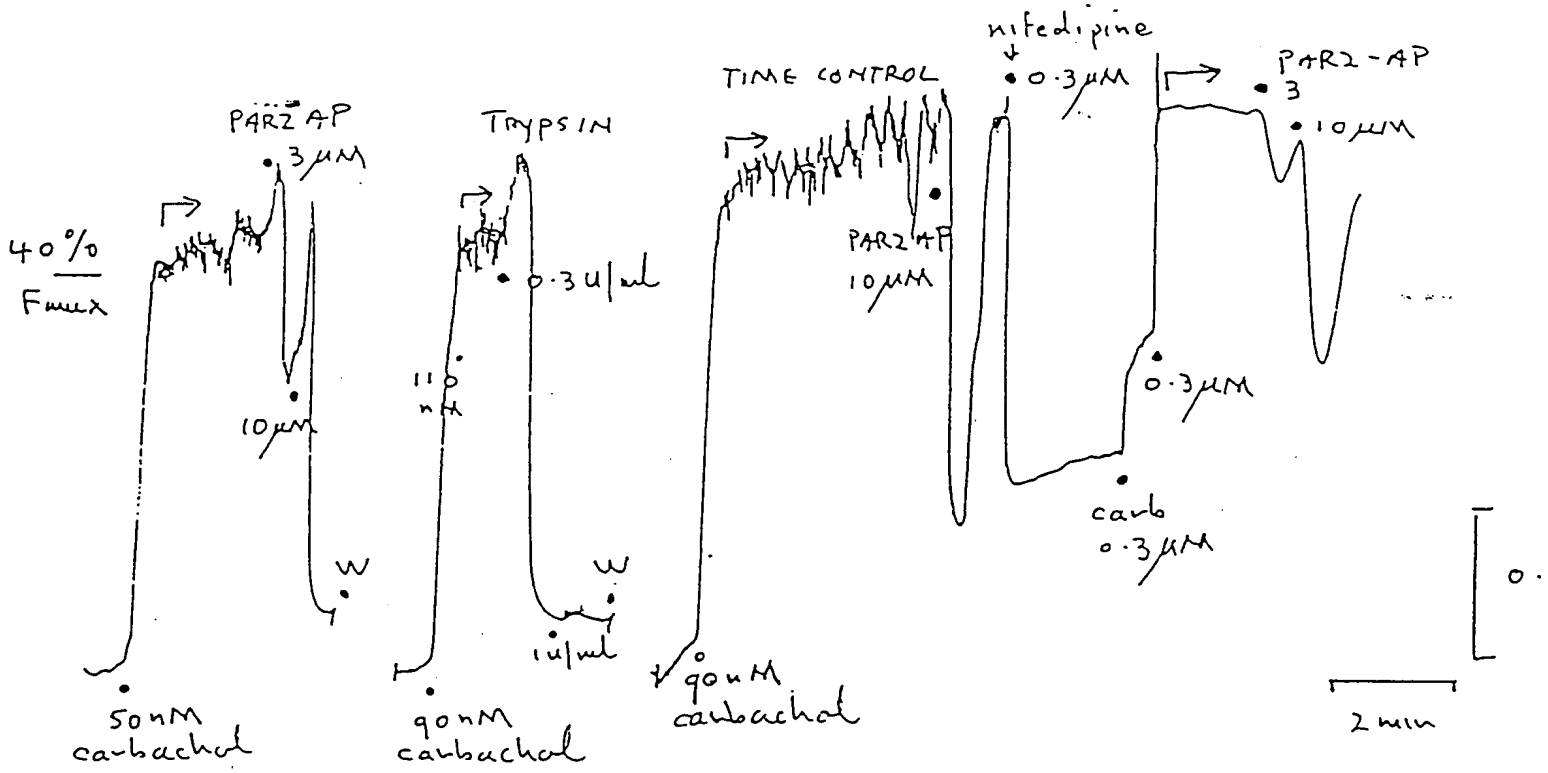

40%
Fmax
50nM
carbachol
PAR2 AP
3μM
10μM
W
90nM
carbachol
TRYPSIN
0·3u/ml
110
nM
1u/ml
W
90nM
carbachol
TIME CONTROL
PAR2 AP
10μM
nifedipine
0·3μM
0·3μM
carb
0·3μM
PAR2-AP
3
10μM
2 min
0·

Figure 4

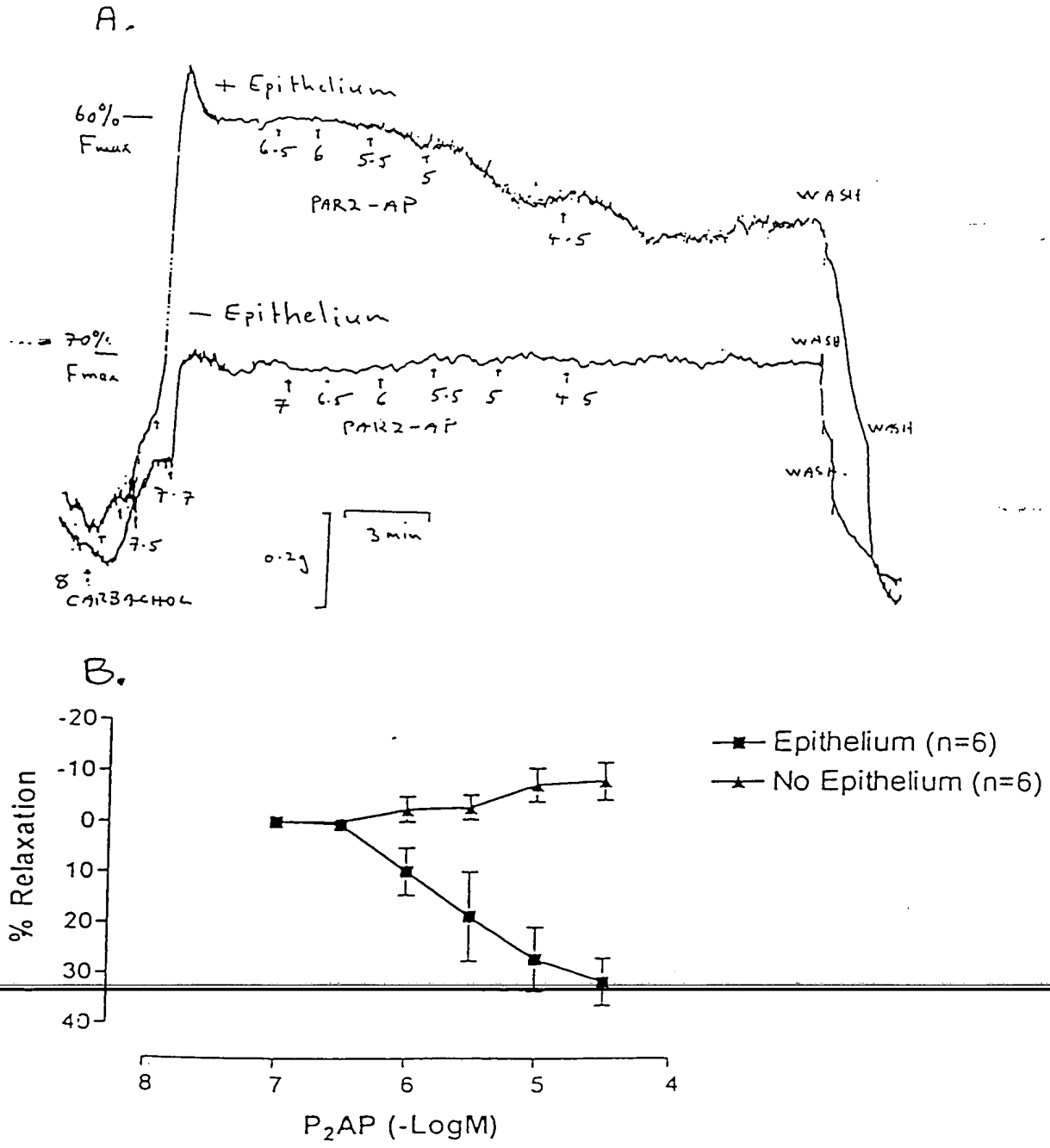

A.
+ Epithelium
60%
Fmax
6.5
6
5.5
5
PAR2-AP
4.5
WASH
— Epithelium
70%
Fmax
7
6.5
6
5.5
5
4.5
PAR2-AP
WASH
WASH
7.7
7.5
8
CARBACHOL
0.2g
3 min
B.
% Relaxation
-20
-10
0
10
20
30
40
Epithelium (n=6)
No Epithelium (n=6)
8
7
6
5
4
P2AP (-LogM)

Figure 5

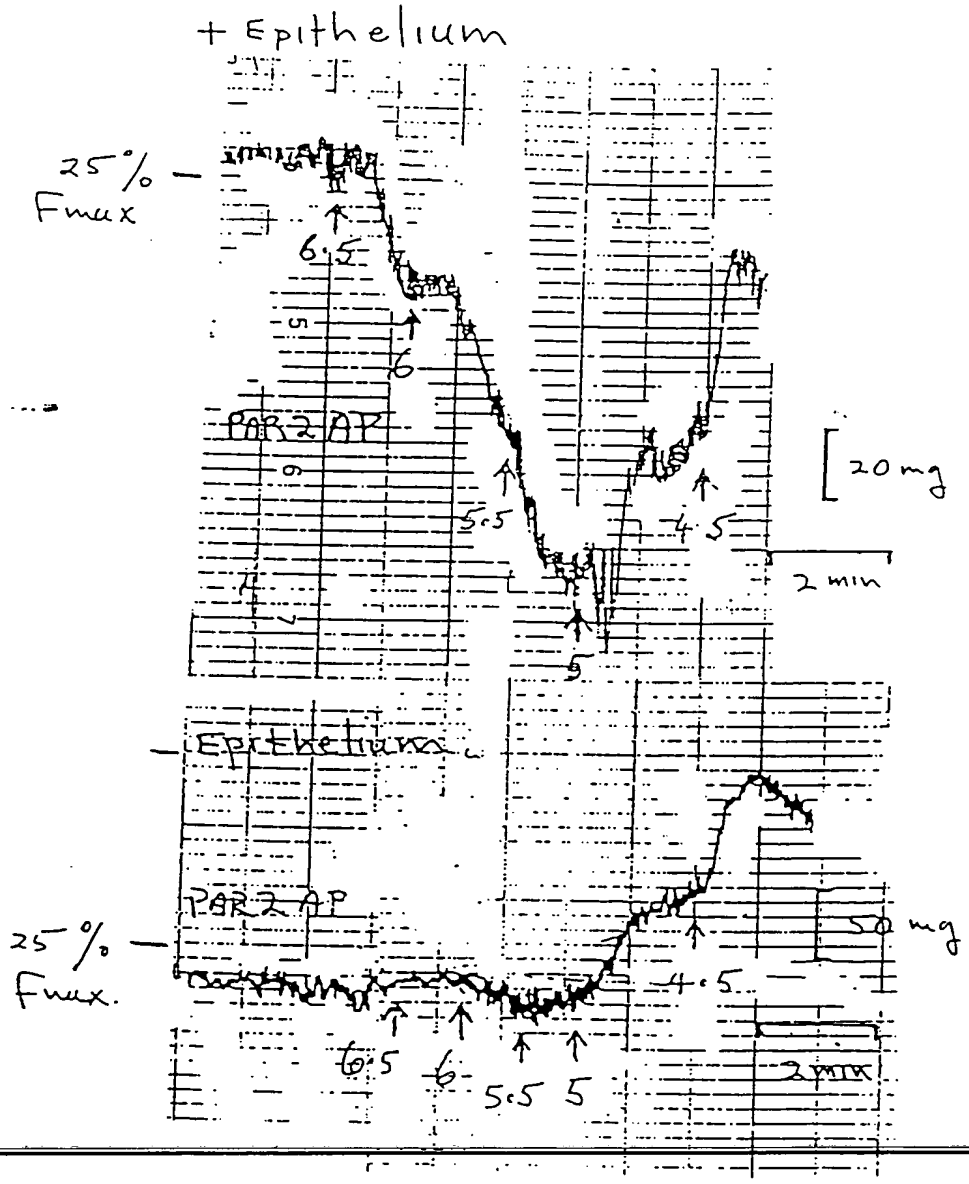

+ Epithelium
25% Fmax
6.5
6
5
PAR2 AP
5.5
4.5
20 mg
2 min
5
- Epithelium
PAR2 AP
25% Fmax.
6.5
6
5.5
5
4.5
50 mg
2 min

Figure 6

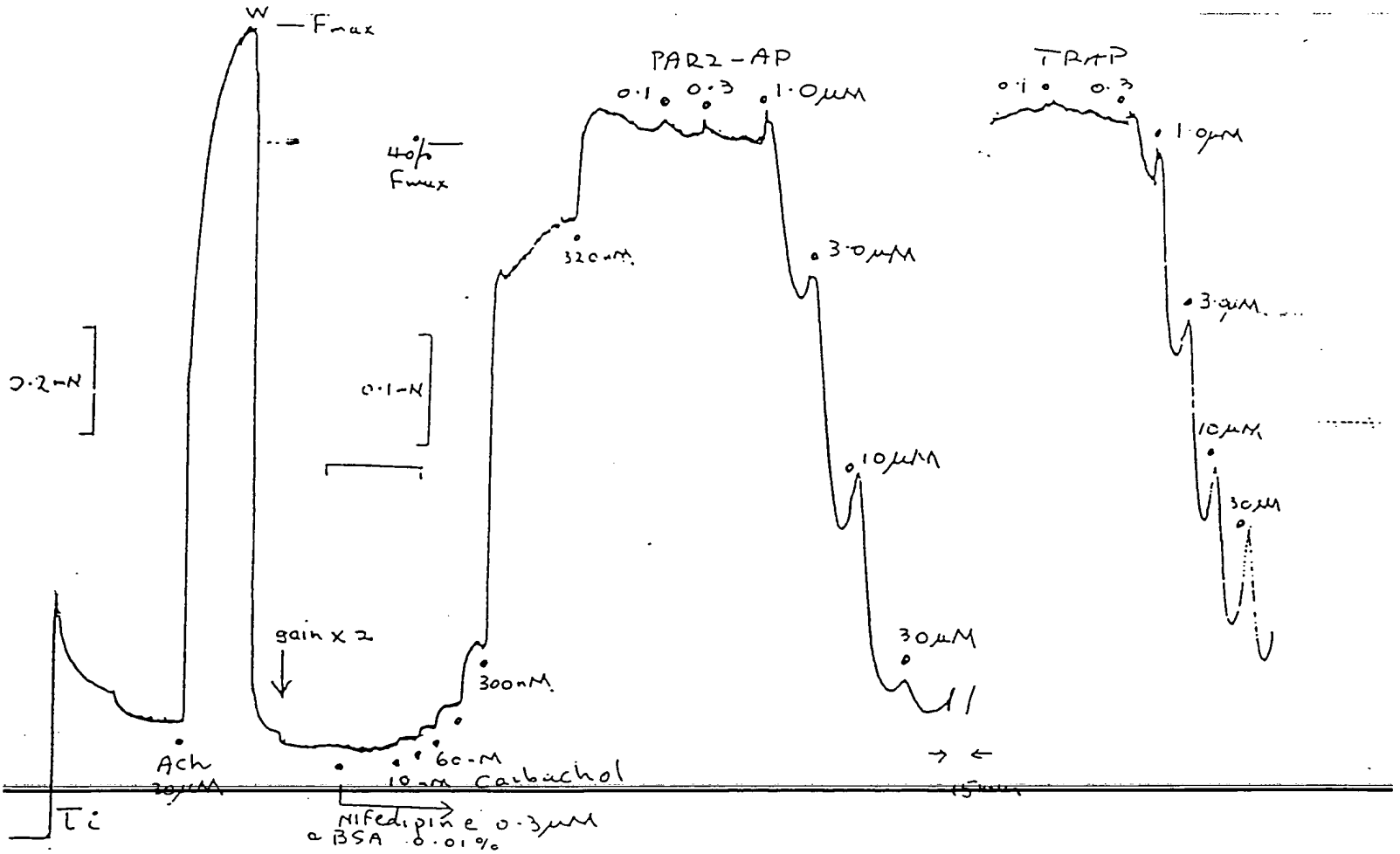

W — Fmax
PAR2-AP
0·1
0·3
1·0μM
TRAP
0·1
0·3
1·0μM
40% Fmax
320nM
3·0μM
3·0μM
0·2-N
0·1-N
10μM
10μM
gain x 2
30μM
30μM
300nM
60-M
10-M Carbachol
Ach
20μM
Ti
Nifedipine 0·3μM
& BSA 0·01%

Figure 7

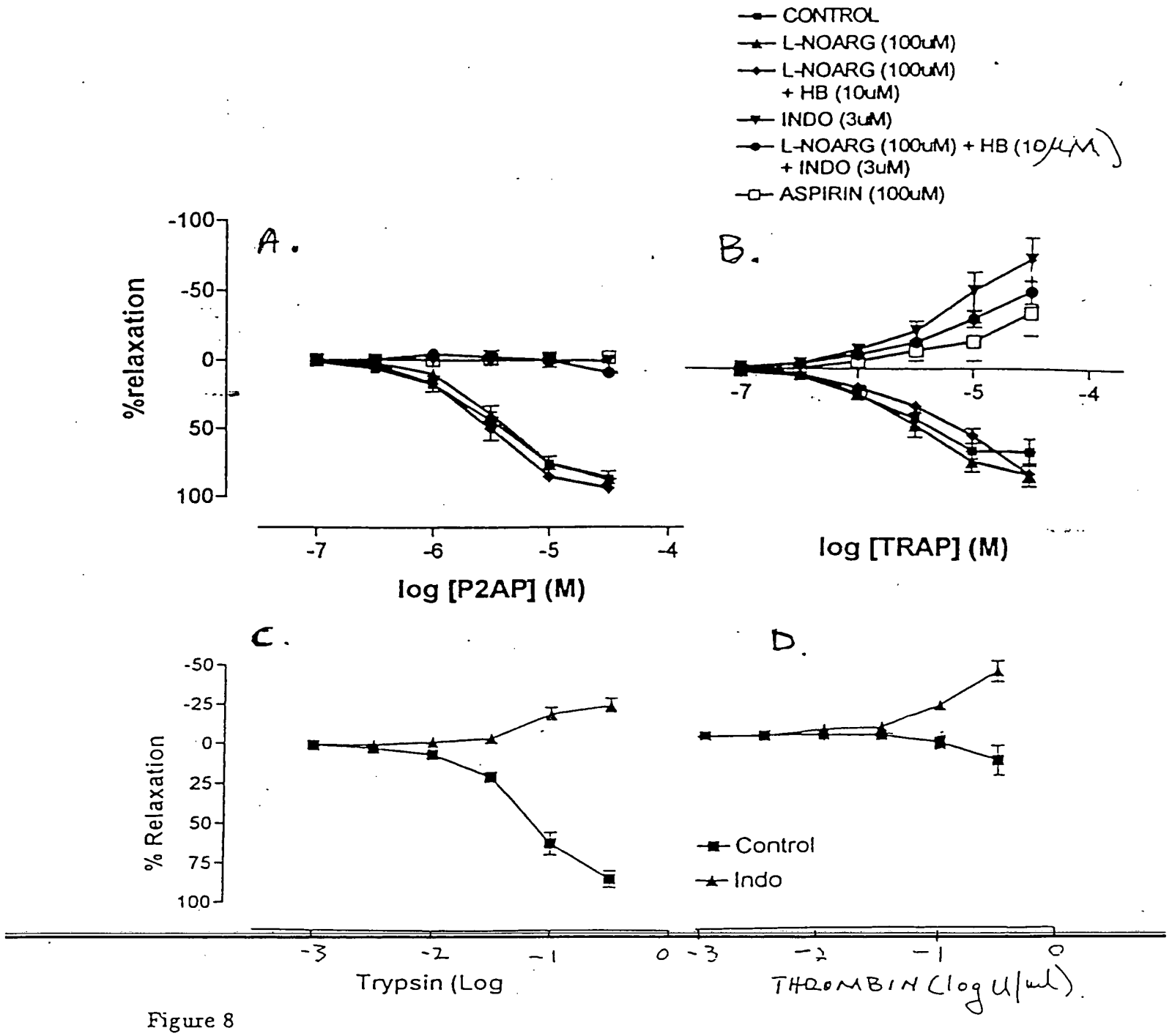


Figure 8

Figure 8

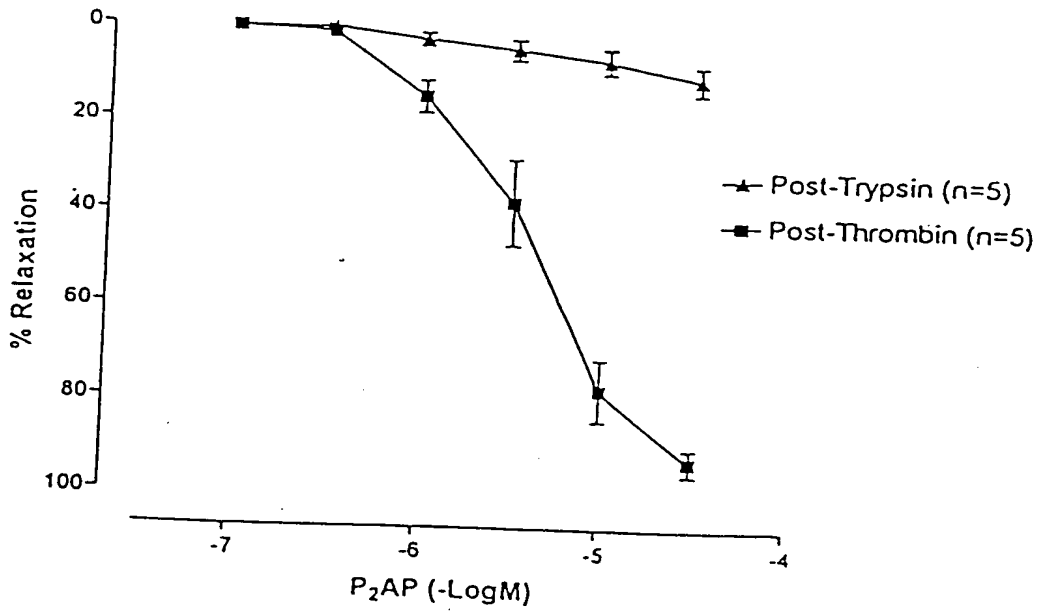

0
20
40
60
80
100
% Relaxation
-7
-6
-5
-4
P2AP (-LogM)
Post-Trypsin (n=5)
Post-Thrombin (n=5)

Figure 9

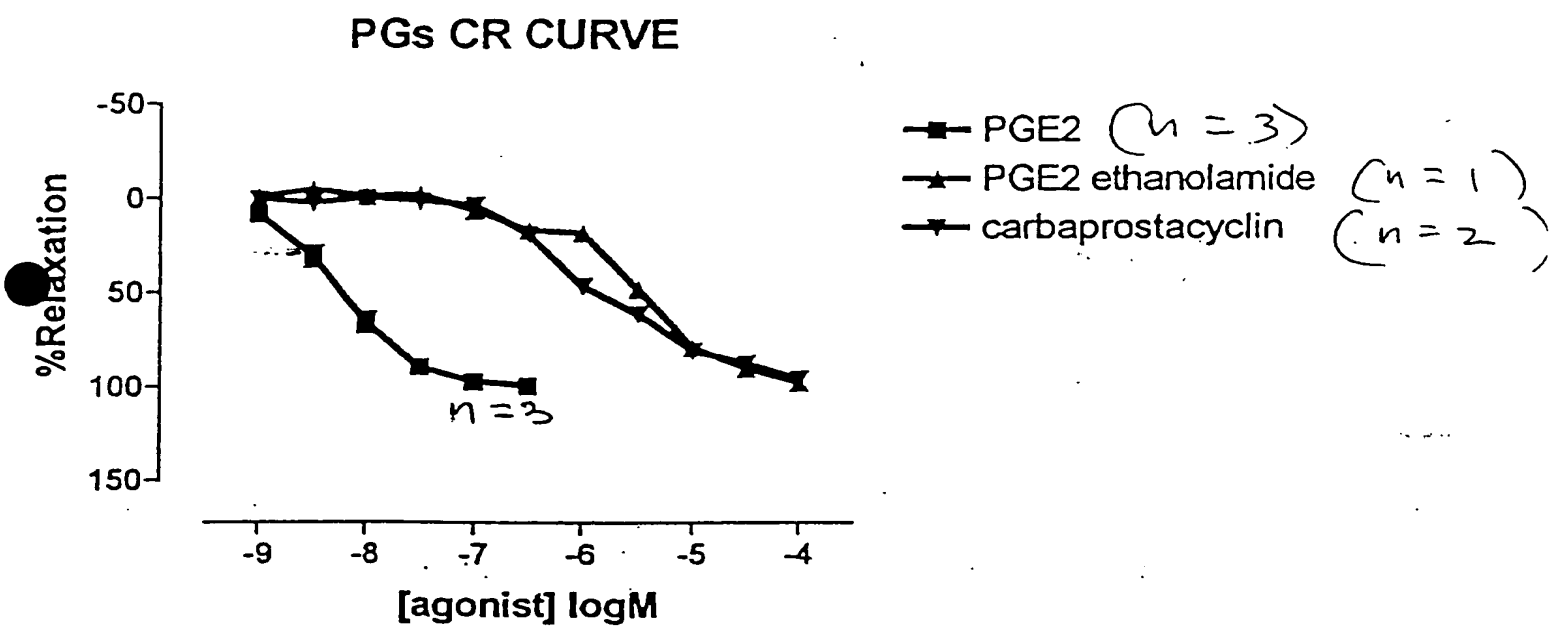

PGs CR CURVE
%Relaxation
-50
0
50
100
150
-9
-8
-7
-6
-5
-4
[agonist] logM
n = 3
PGE2 (n = 3)
PGE2 ethanolamide (n = 1)
carbaprostacyclin (n = 2)

Figure 10

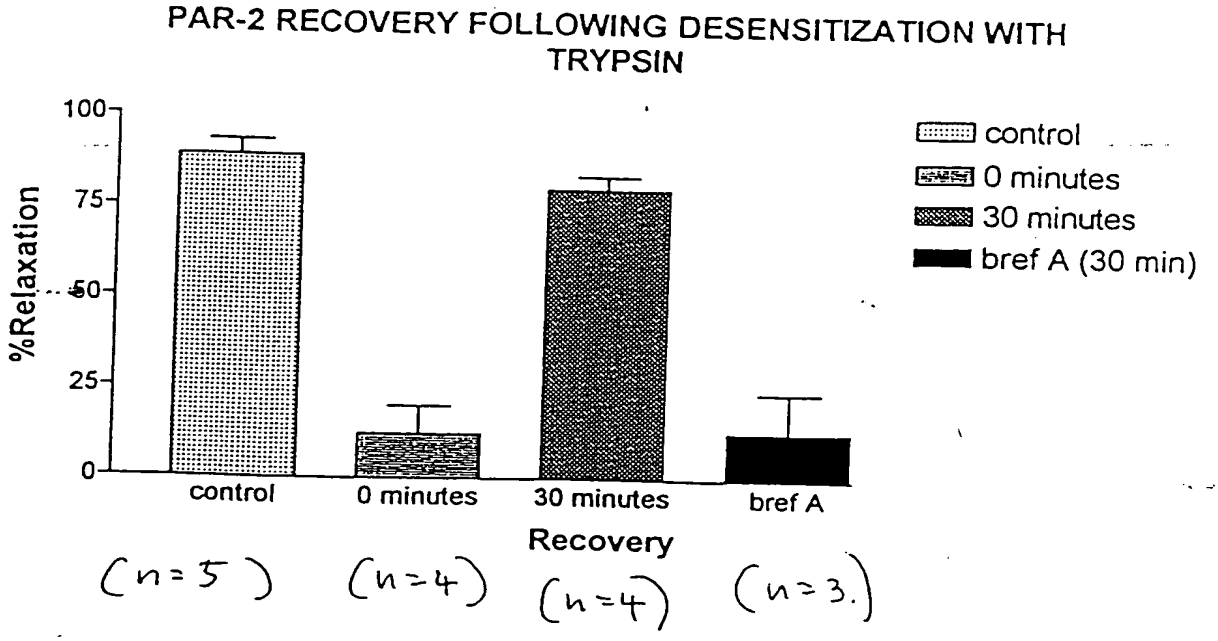

PAR-2 RECOVERY FOLLOWING DESENSITIZATION WITH TRYPSIN
%Relaxation
100
75
50
25
0
control
0 minutes
30 minutes
bref A
Recovery
(n=5)
(n=4)
(n=4)
(n=3.)
control
0 minutes
30 minutes
bref A (30 min)

Figure 11A

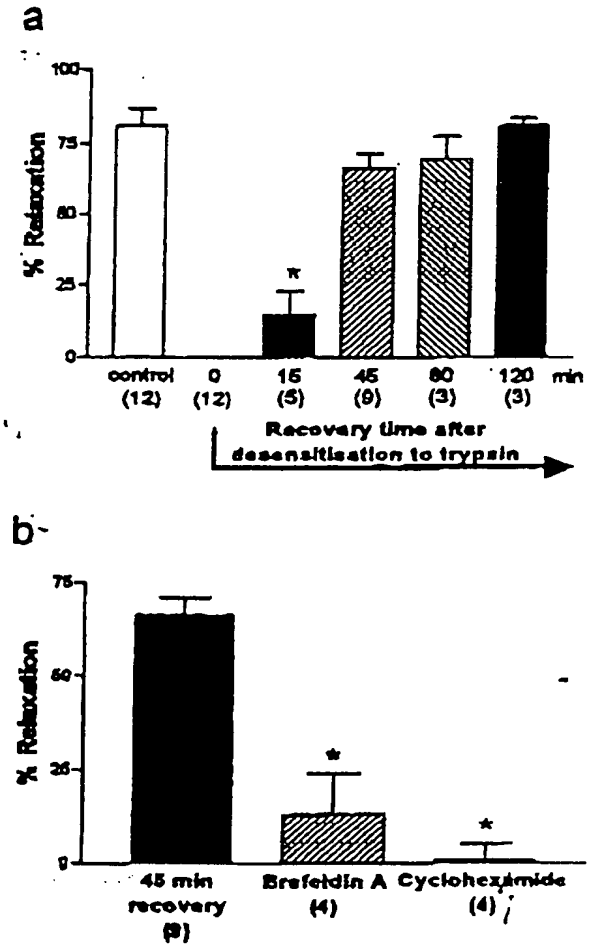

a
100
75
50
25
0
% Relaxation
*
control (12)
0 (12)
15 (5)
45 (9)
80 (3)
120 (3)
min
Recovery time after desensitisation to trypsin
b
75
50
25
0
% Relaxation
*
*
45 min recovery (9)
Brefeldin A (4)
Cycloheximide (4)

Figure 11b

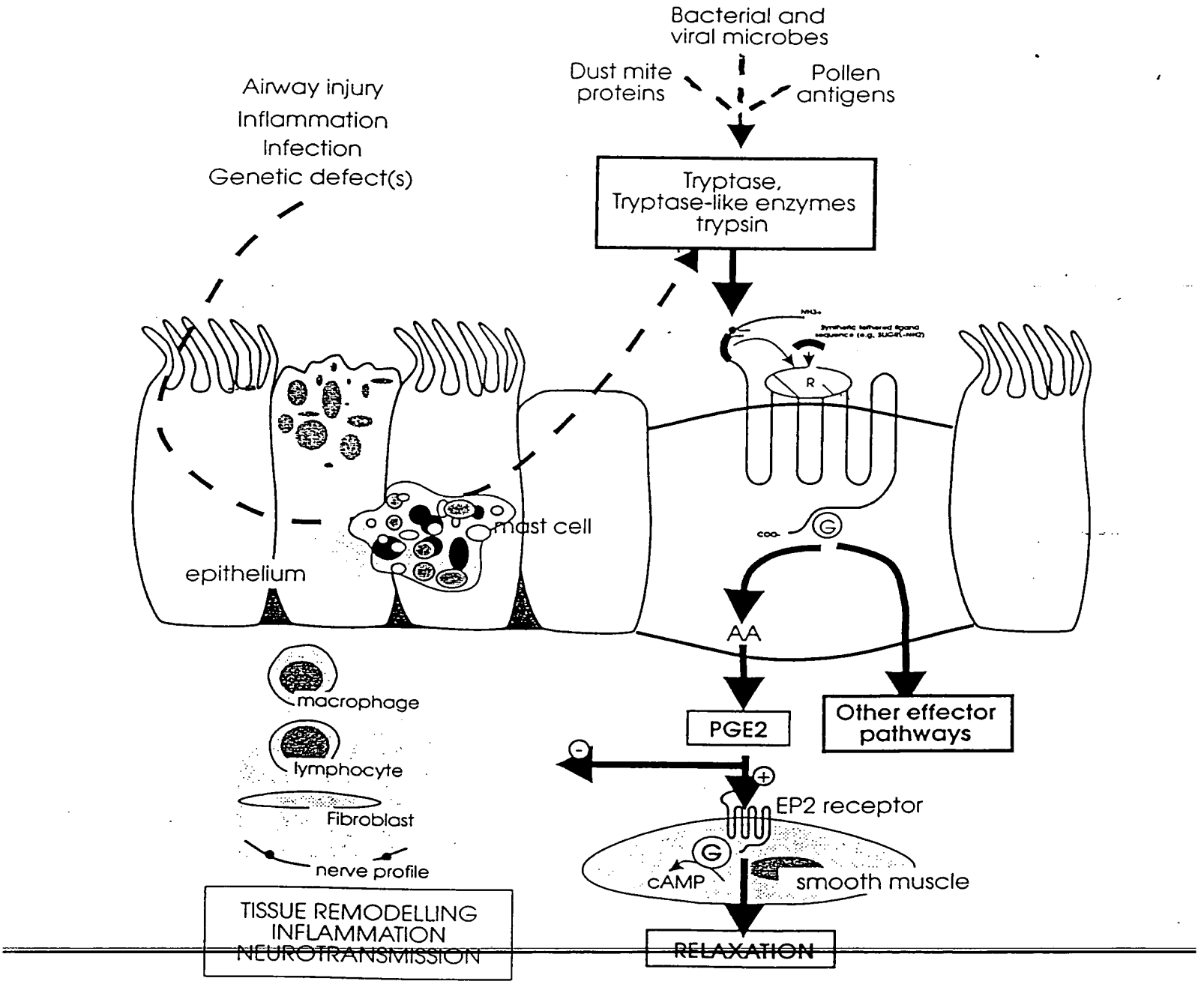

Bacterial and
viral microbes
Dust mite
proteins
Pollen
antigens
Airway injury
Inflammation
Infection
Genetic defect(s)
Tryptase,
Tryptase-like enzymes
trypsin
R
mast cell
epithelium
AA
macrophage
lymphocyte
Fibroblast
nerve profile
PGE2
Other effector
pathways
EP2 receptor
cAMP
smooth muscle
TISSUE REMODELLING
INFLAMMATION
NEUROTRANSMISSION
RELAXATION

Figure 12

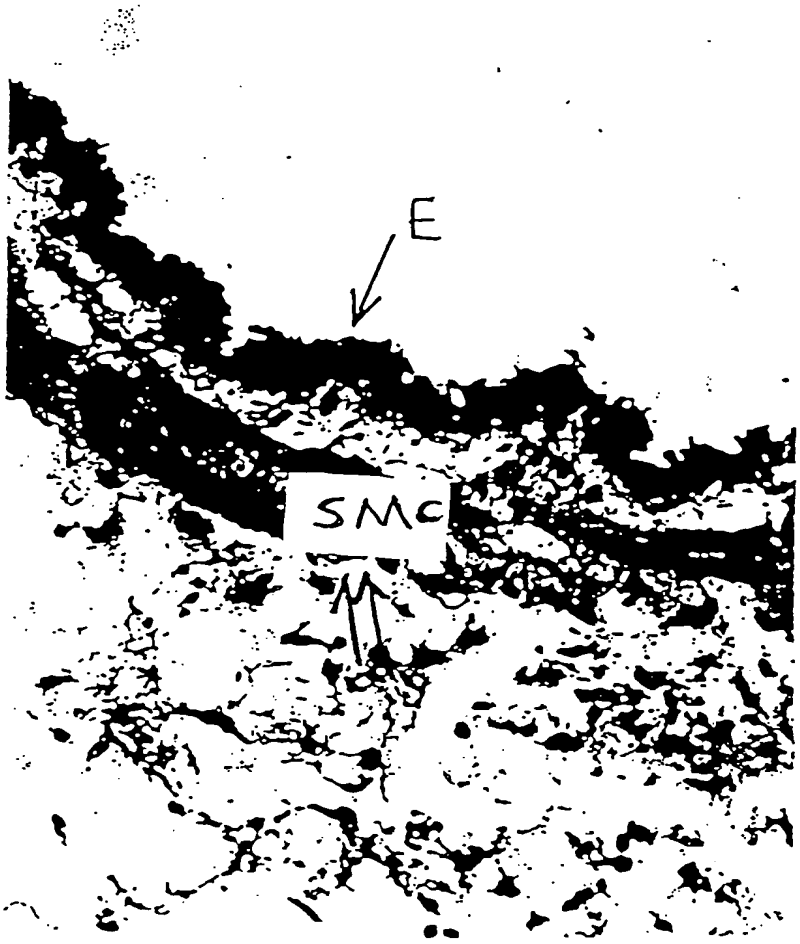


Figure 13

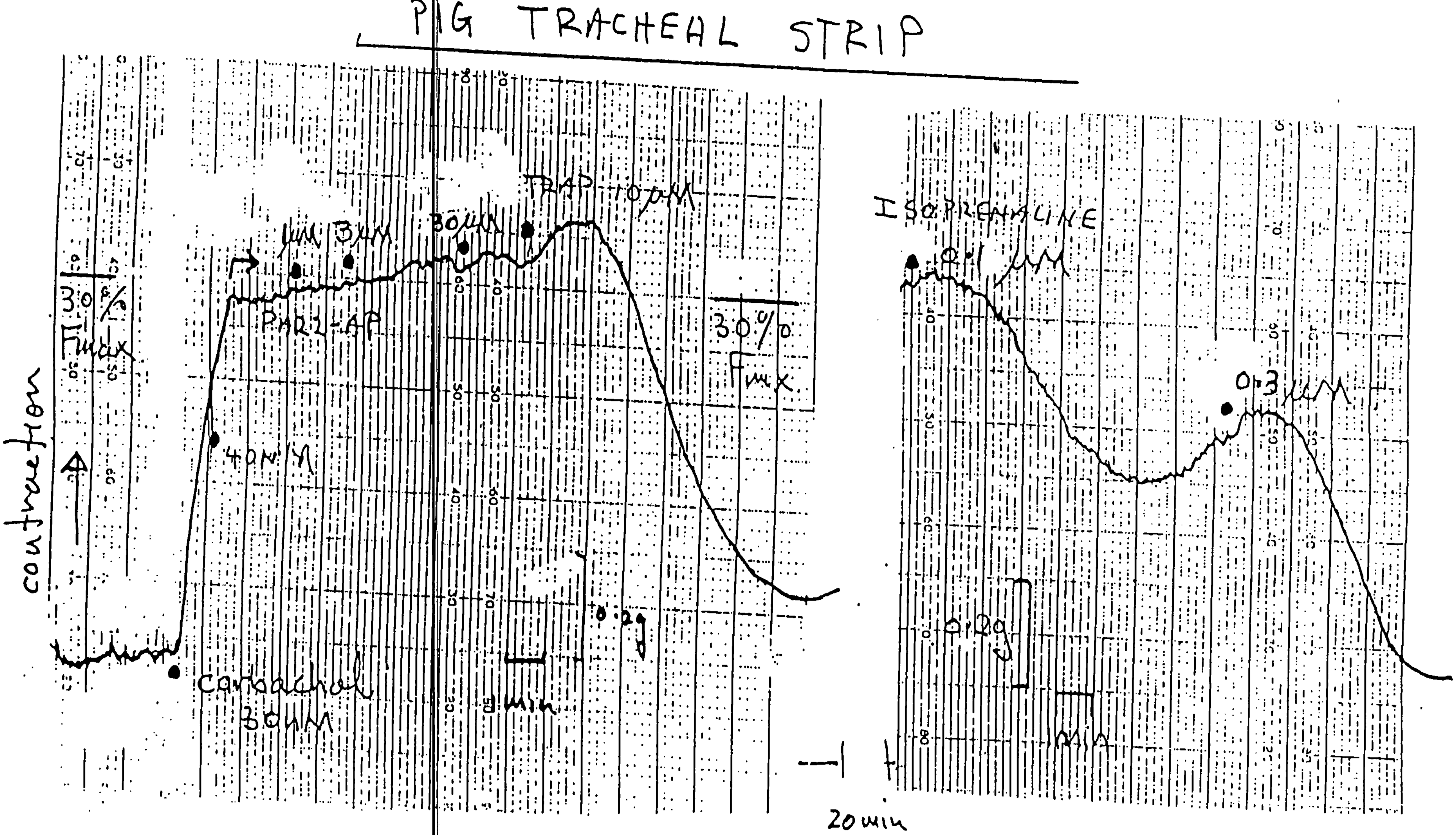

PIG TRACHEAL STRIP
contraction
30% Fmax
carbachol 30nM
40nM
PAR2-AP
1μM 3μM 30μM
TRAP 10μM
30% Fmax
0.2g
5 min
ISOPRENALINE 0.1 μM
0.3 μM
0.2g
1 MIN
20 min

Figure 14

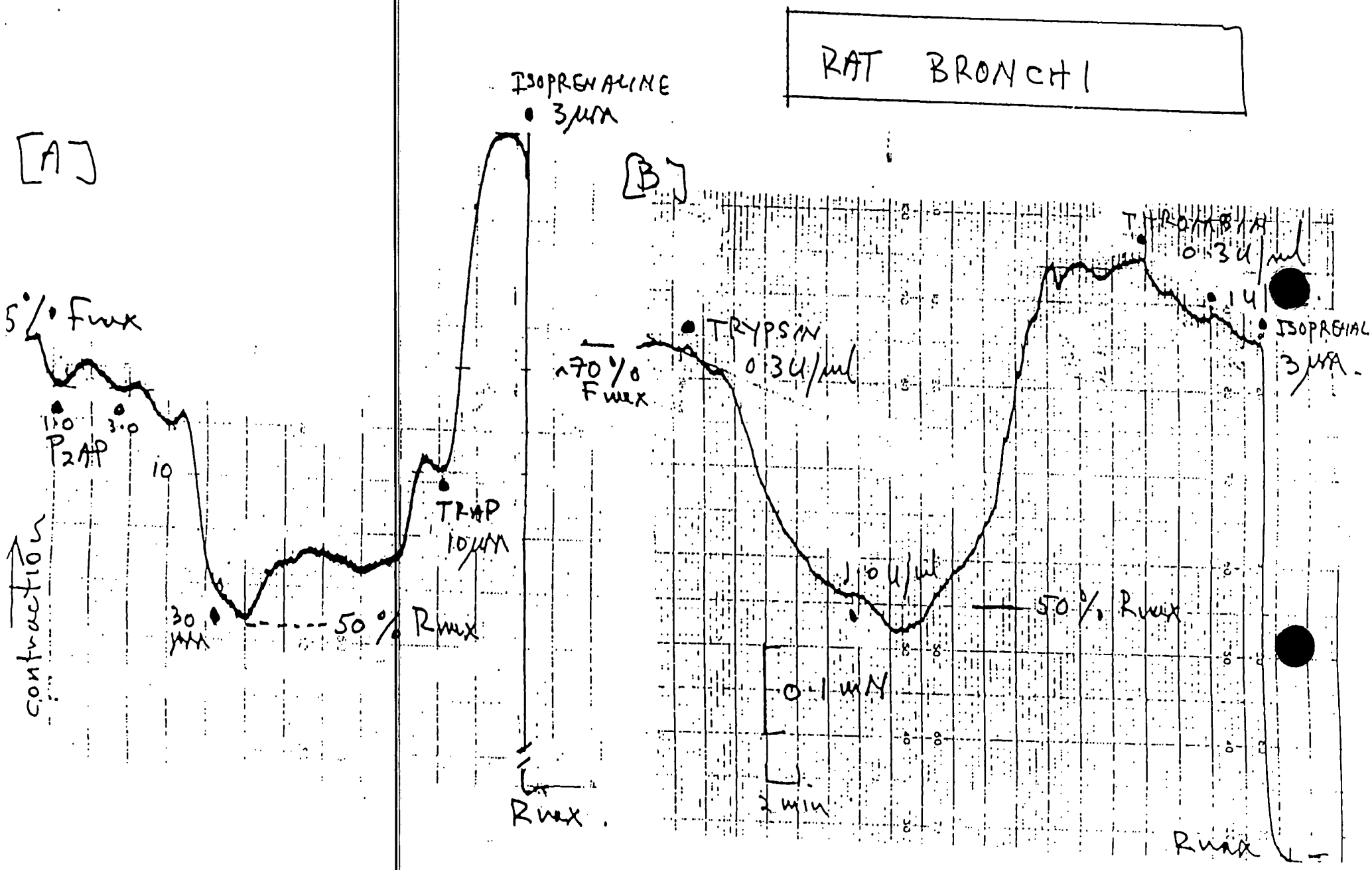

RAT BRONCHI
[A]
[B]
ISOPRENALINE
3 μM
5% Fmax
1.0
P2AP
3.0
10
30
μM
50 % Rmax
TRAP
10 μM
Rmax
contraction
TRYPSIN
0.3 U/ml
~70 %
Fmax
1.0 U/ml
50 % Rmax
0.1 mN
2 min
TRYPSIN
0.3 U/ml
1.4
ISOPRENAL
3 μM
Rmax

Figure 15

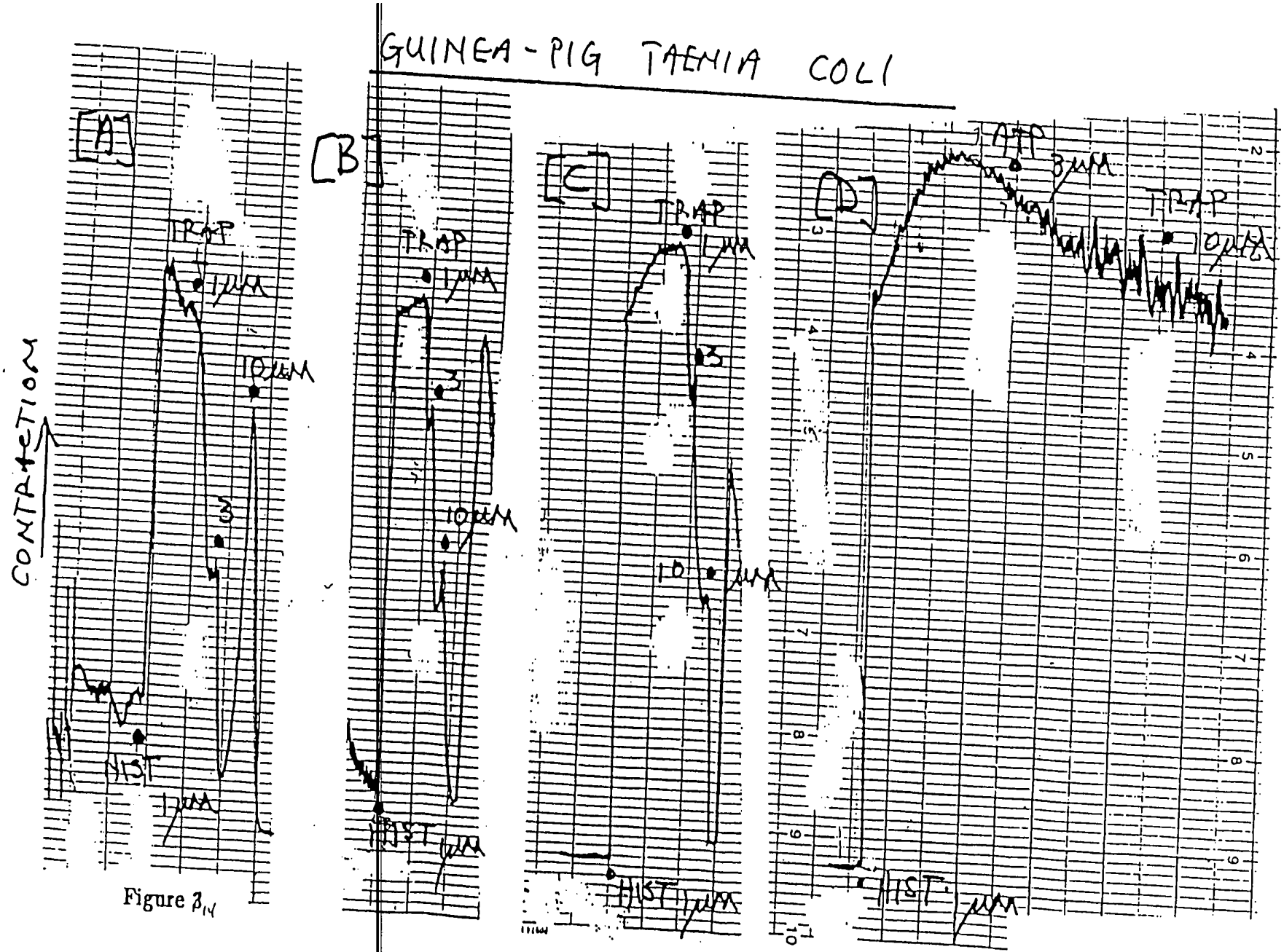


Figure 3_{14}

Figure 16

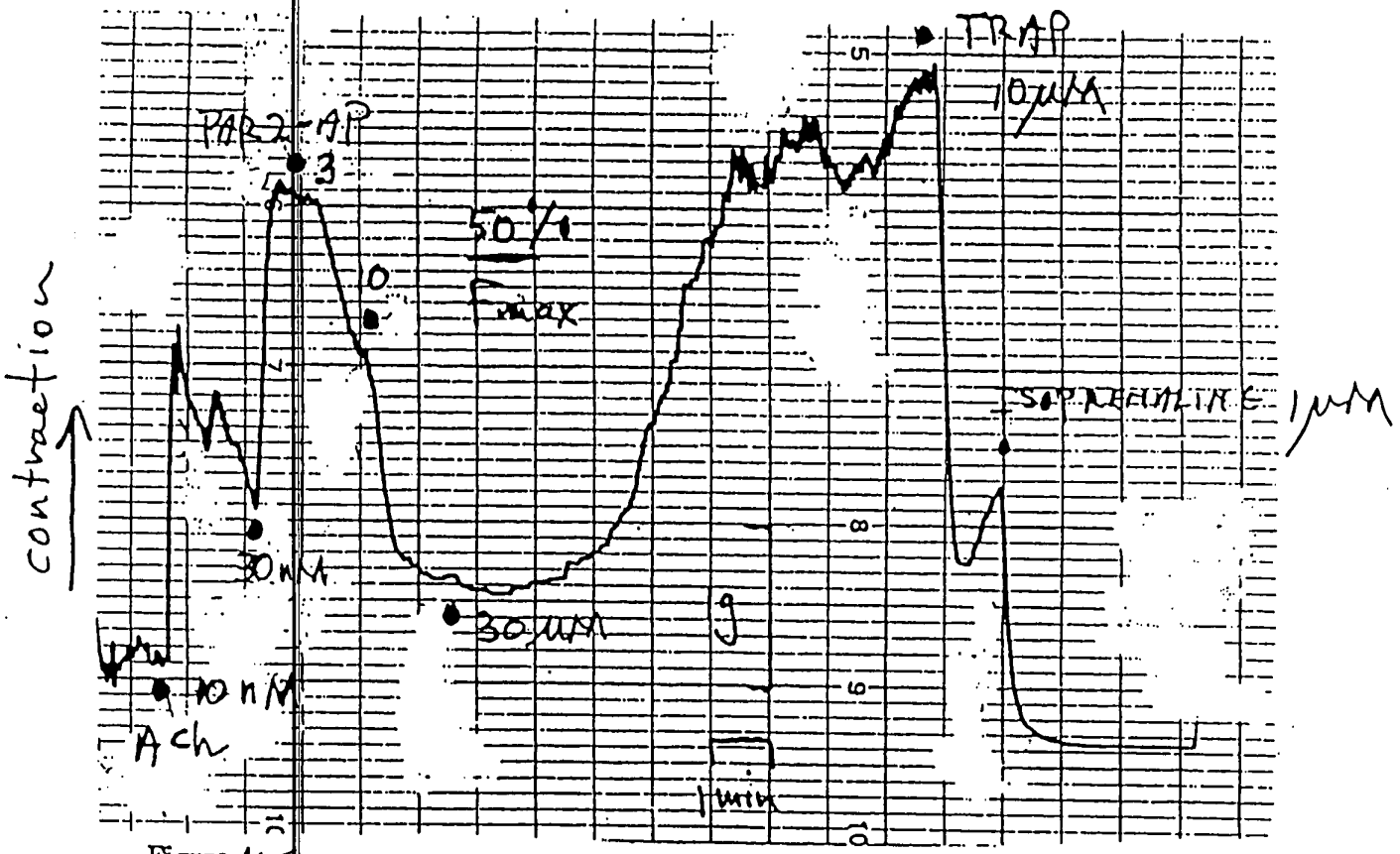


Figure A-15

Figure 17

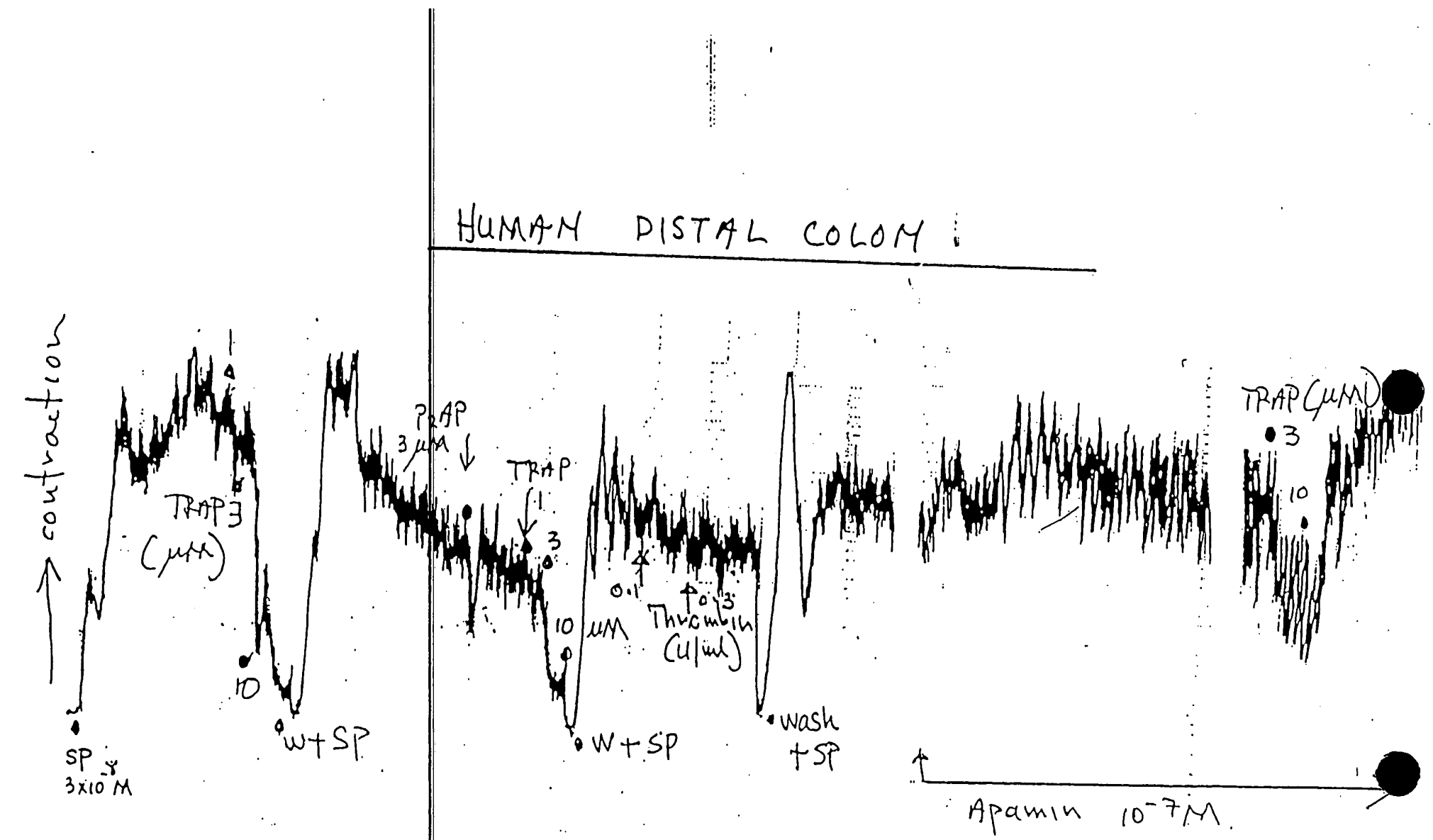

HUMAN DISTAL COLON
contraction
TRAP3 (μM)
10
W+SP
SP 3x10⁻⁸M
PrAP 3μM
TRAP 1
3
10 μM
W+SP
0.1
0.3
Thrombin (U/ml)
wash +SP
TRAP (μM)
3
10
Apamin 10⁻⁷M

Figure 18

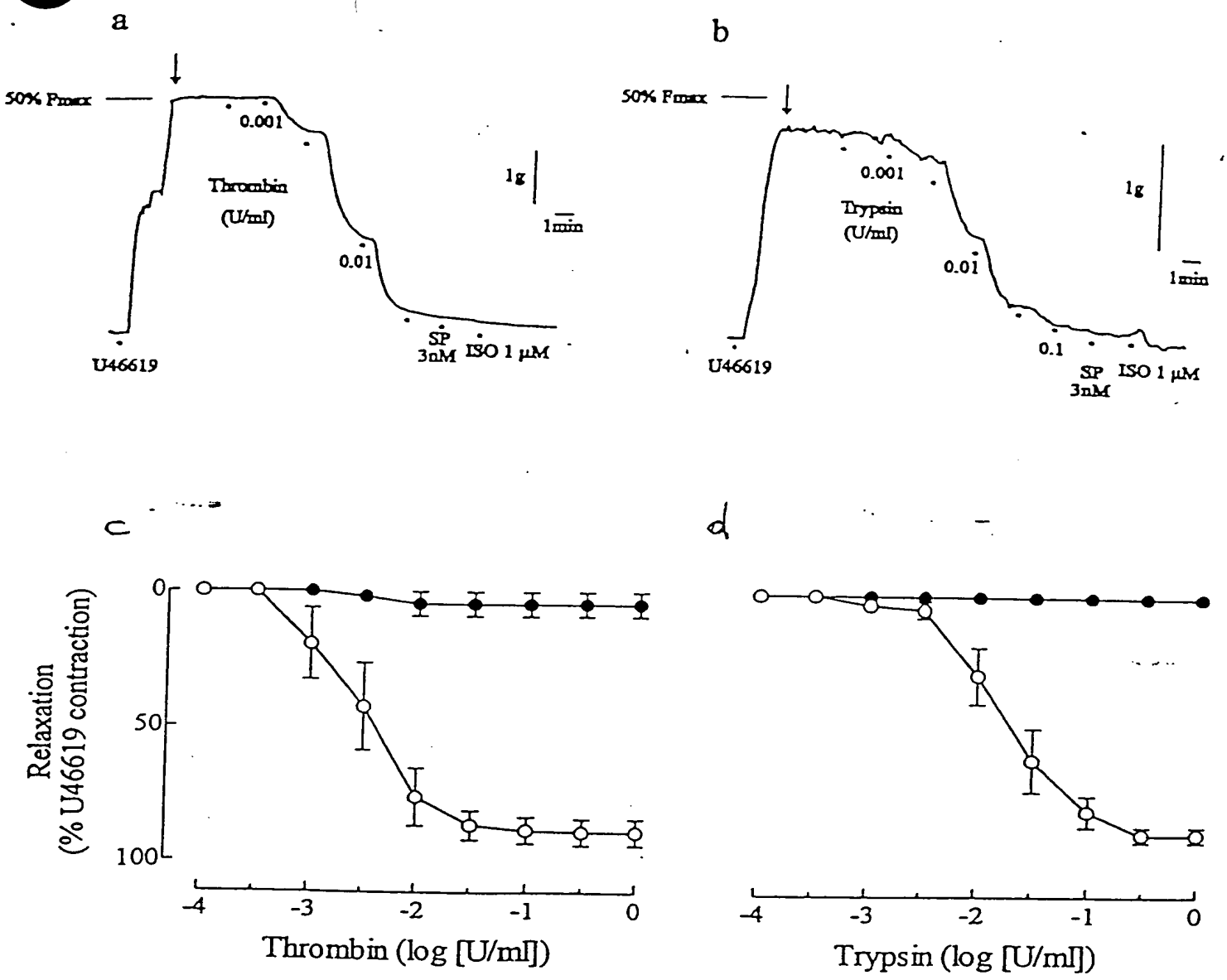

a
50% Pmax
0.001
Thrombin
(U/ml)
0.01
1g
1min
U46619
SP
3nM
ISO 1 μM
b
50% Pmax
0.001
Trypsin
(U/ml)
0.01
0.1
1g
1min
U46619
SP
3nM
ISO 1 μM
c
Relaxation
(% U46619 contraction)
0
50
100
-4
-3
-2
-1
0
Thrombin (log [U/ml])
d
-4
-3
-2
-1
0
Trypsin (log [U/ml])

Figure 19

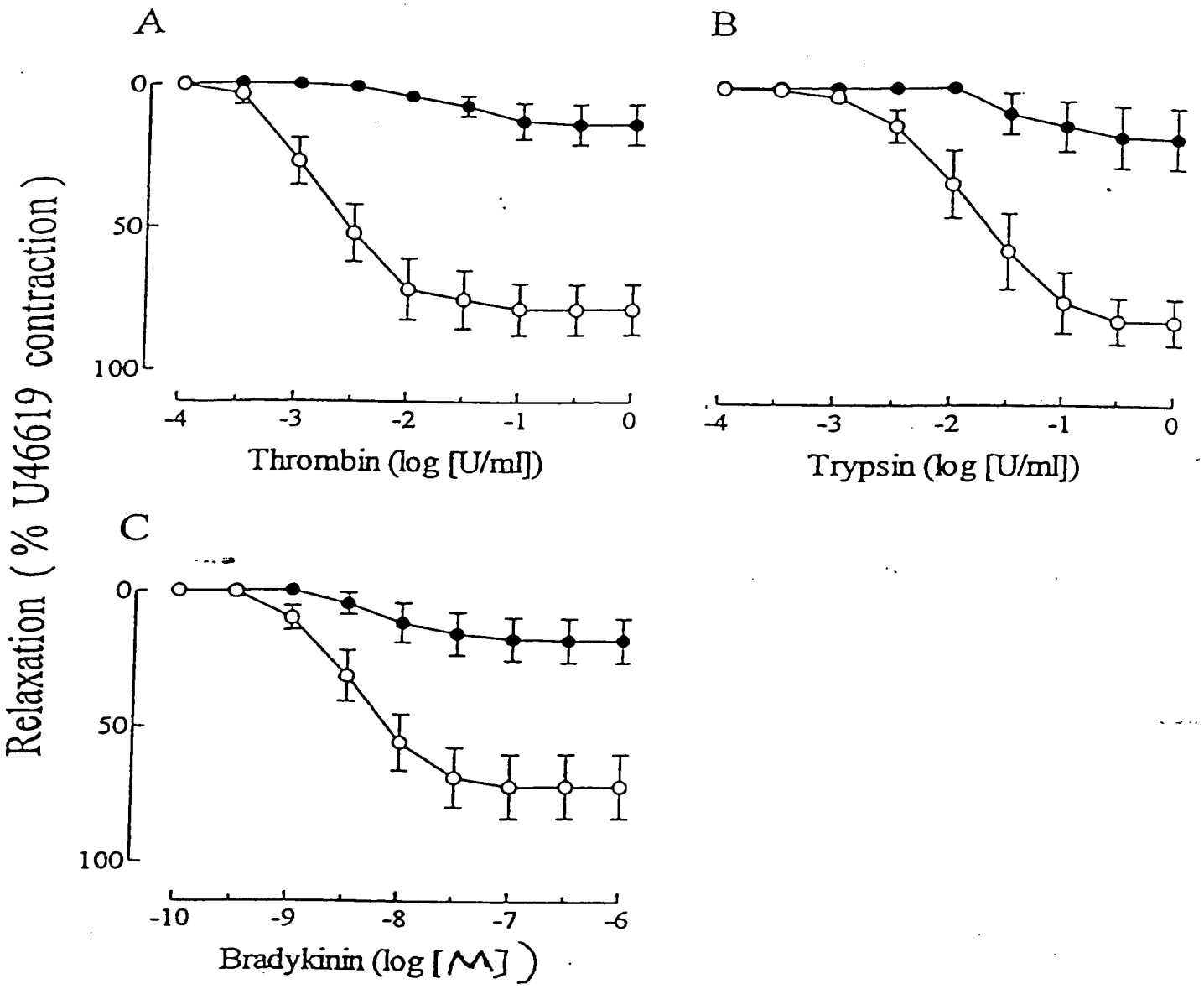

A
B
C
Relaxation (% U46619 contraction)
0
50
100
-4
-3
-2
-1
0
Thrombin (log [U/ml])
Trypsin (log [U/ml])
-10
-9
-8
-7
-6
Bradykinin (log [M])

Figure 20

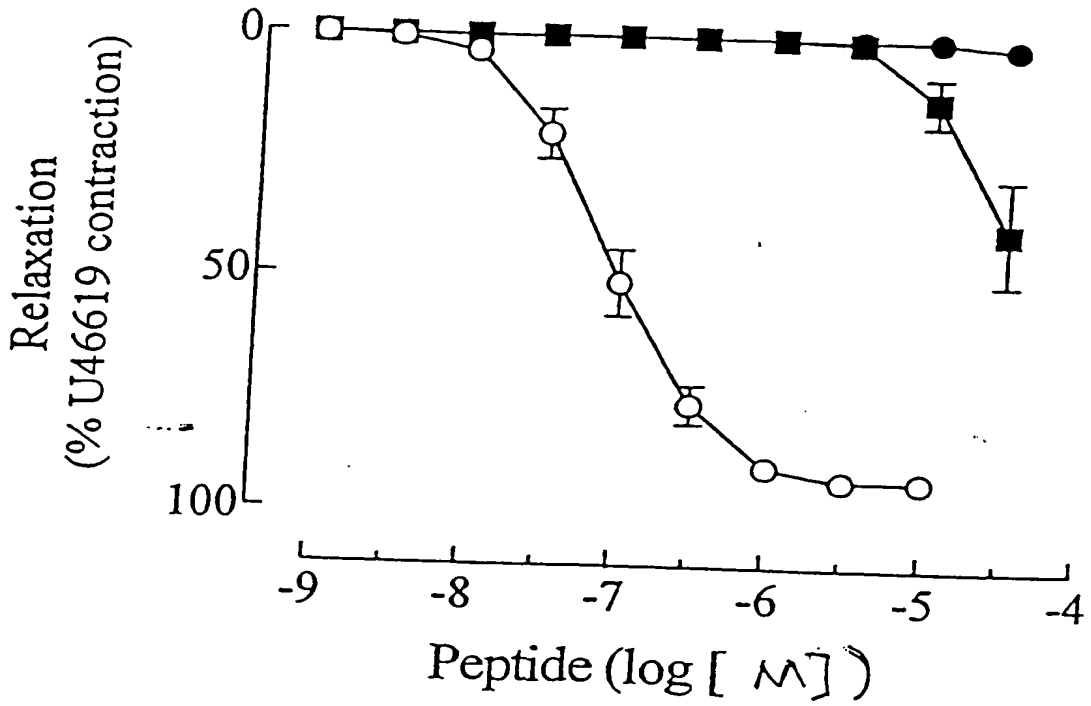

Relaxation
(% U46619 contraction)
0
50
100
-9
-8
-7
-6
-5
-4
Peptide (log [M])

Figure 21

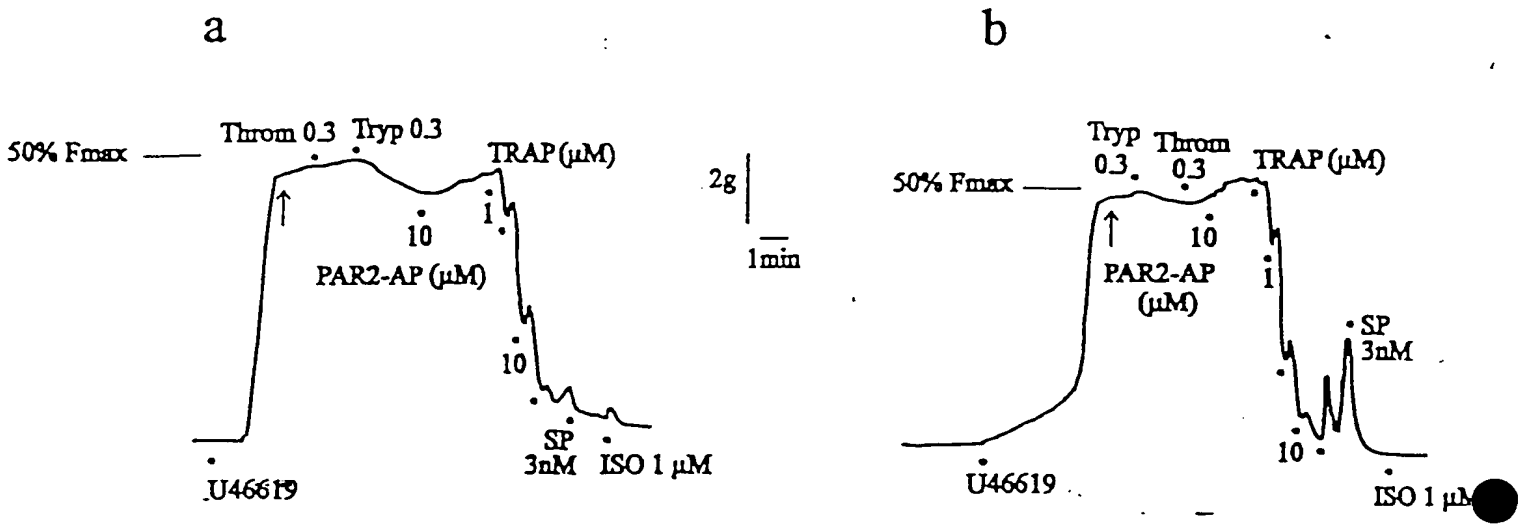

a
b
50% Fmax
Throm 0.3
Tryp 0.3
TRAP (µM)
10
PAR2-AP (µM)
1
10
SP
3nM
ISO 1 µM
U46619
2g
1min
Tryp
0.3
Throm
0.3
TRAP (µM)
50% Fmax
10
PAR2-AP
(µM)
1
SP
3nM
10
U46619
ISO 1 µM

Figure 22

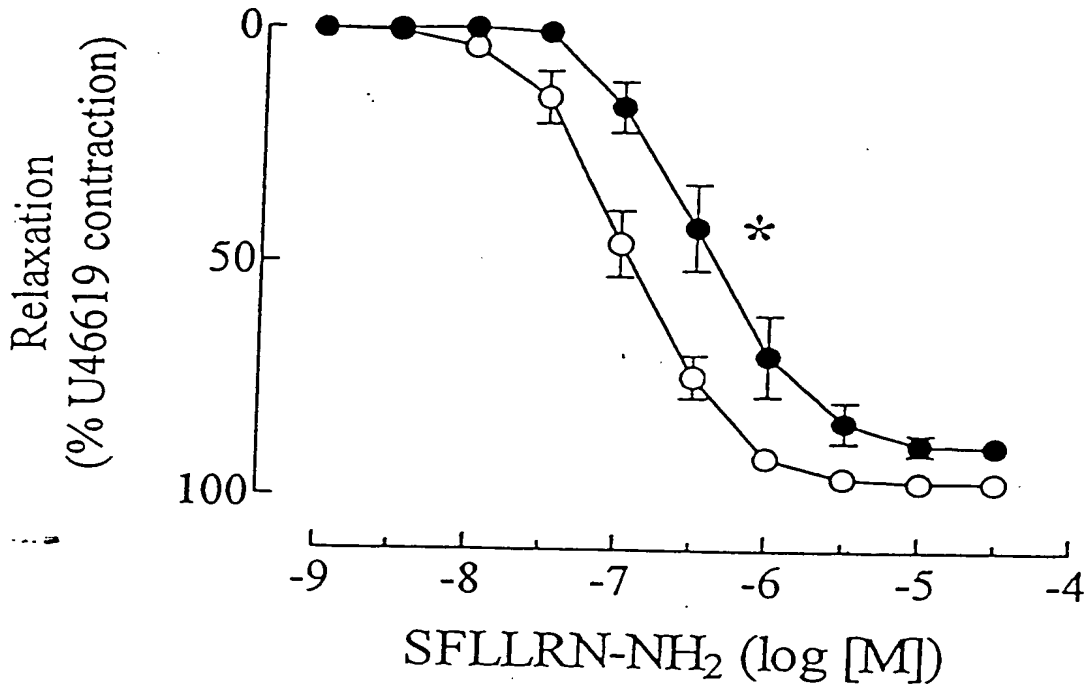

Relaxation
(% U46619 contraction)
0
50
100
*
-9
-8
-7
-6
-5
-4
SFLLRN-NH2 (log [M])

Figure 23

FIGURE 24

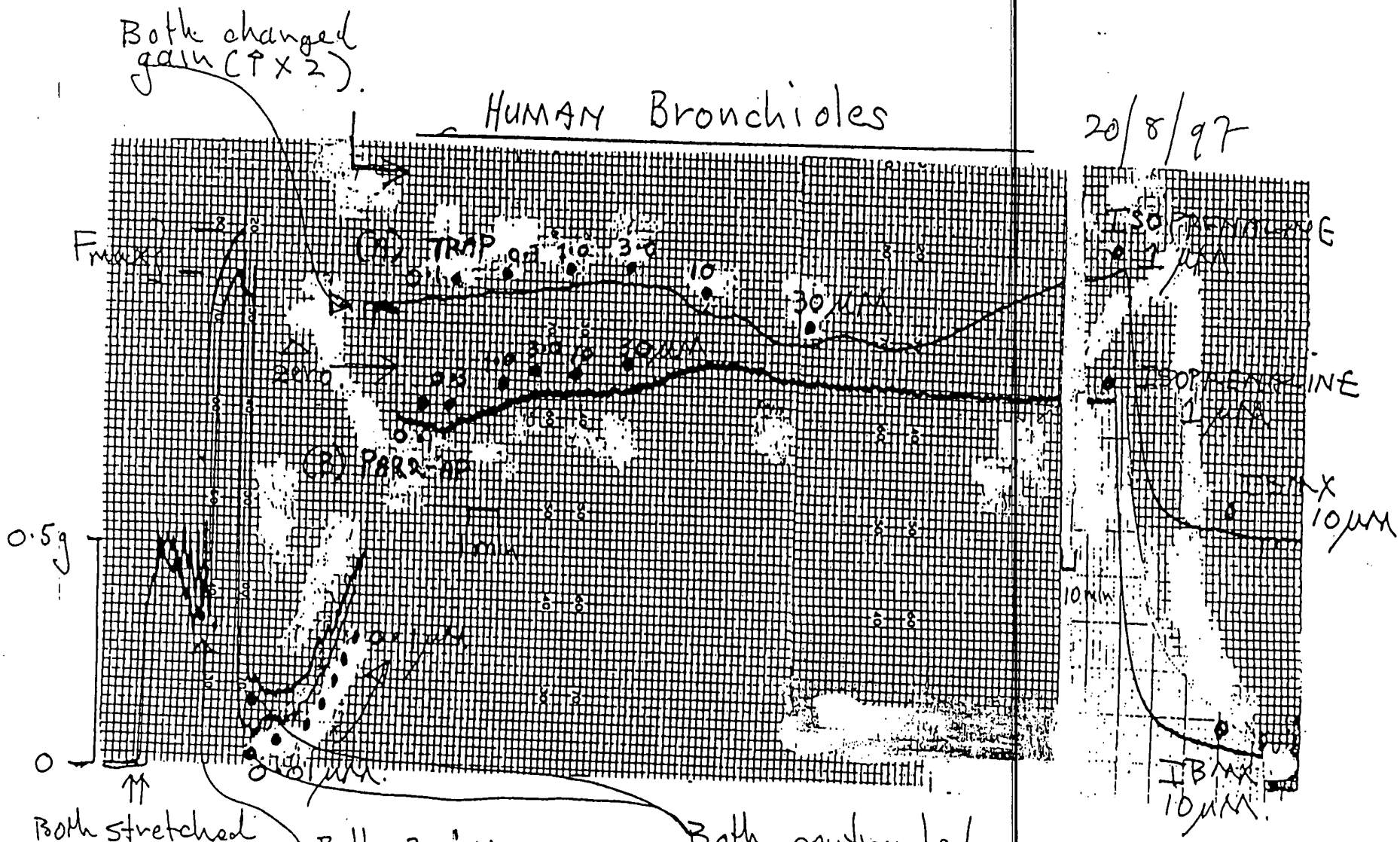
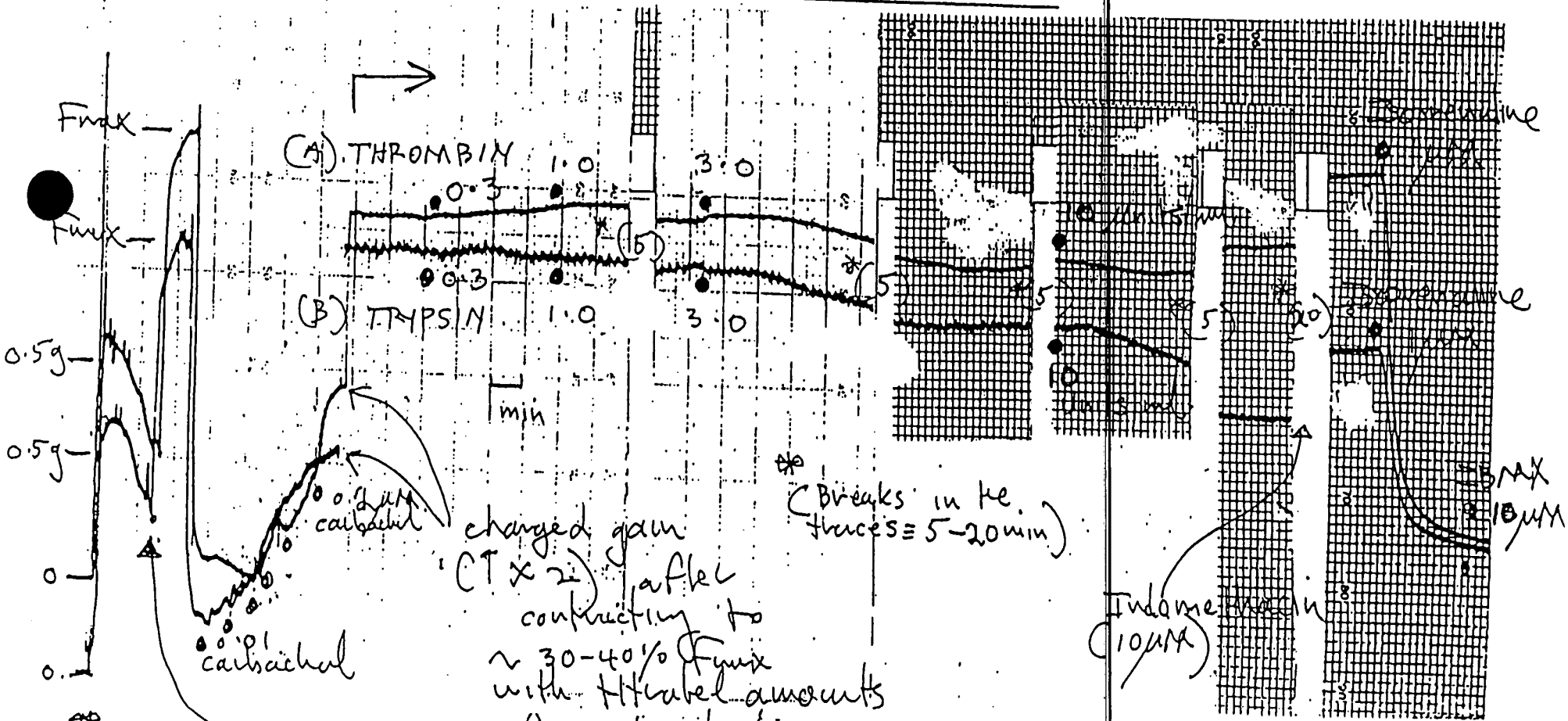

Both changed gain (↑ x 2).
HUMAN Bronchioles
20/8/97
Fmax
(A) TRAP
(B) P8R2-AP
ISOPRENALINE
ISOPRENALINE
1 μM
10 μM
IBMX
10 μM
0.5g
0
Both stretched to 0.5g passive force.
Both 30 μM acetylcholine (Fmax)
Both contracted to ~30 Fmax with titrated concentrations of carbachol. Also 0.005% BSA was added

FIGURE 05

20/8/97

Human Bronchioles



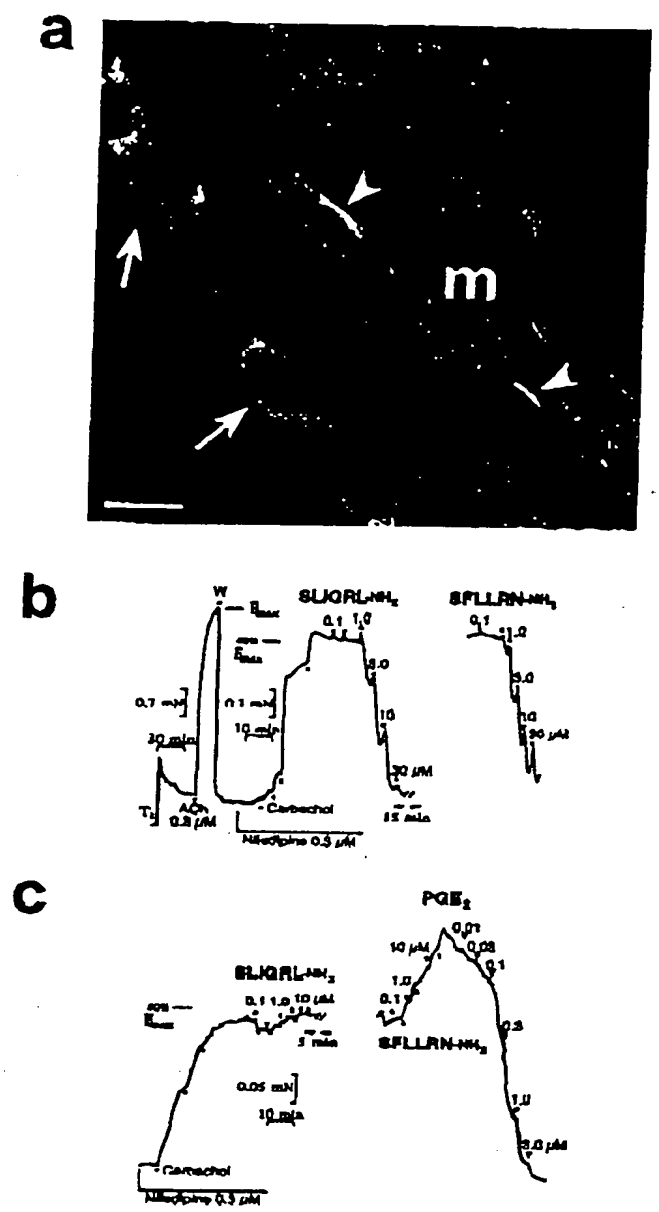

a
m
b
W
SLIQRL-NH2
SFLLRN-NH2
Carbachol
Nifedipine 0.3 μM
c
PGE2
SLIQRL-NH2
SFLLRN-NH2
Carbachol
Nifedipine 0.3 μM
d

Figure 26

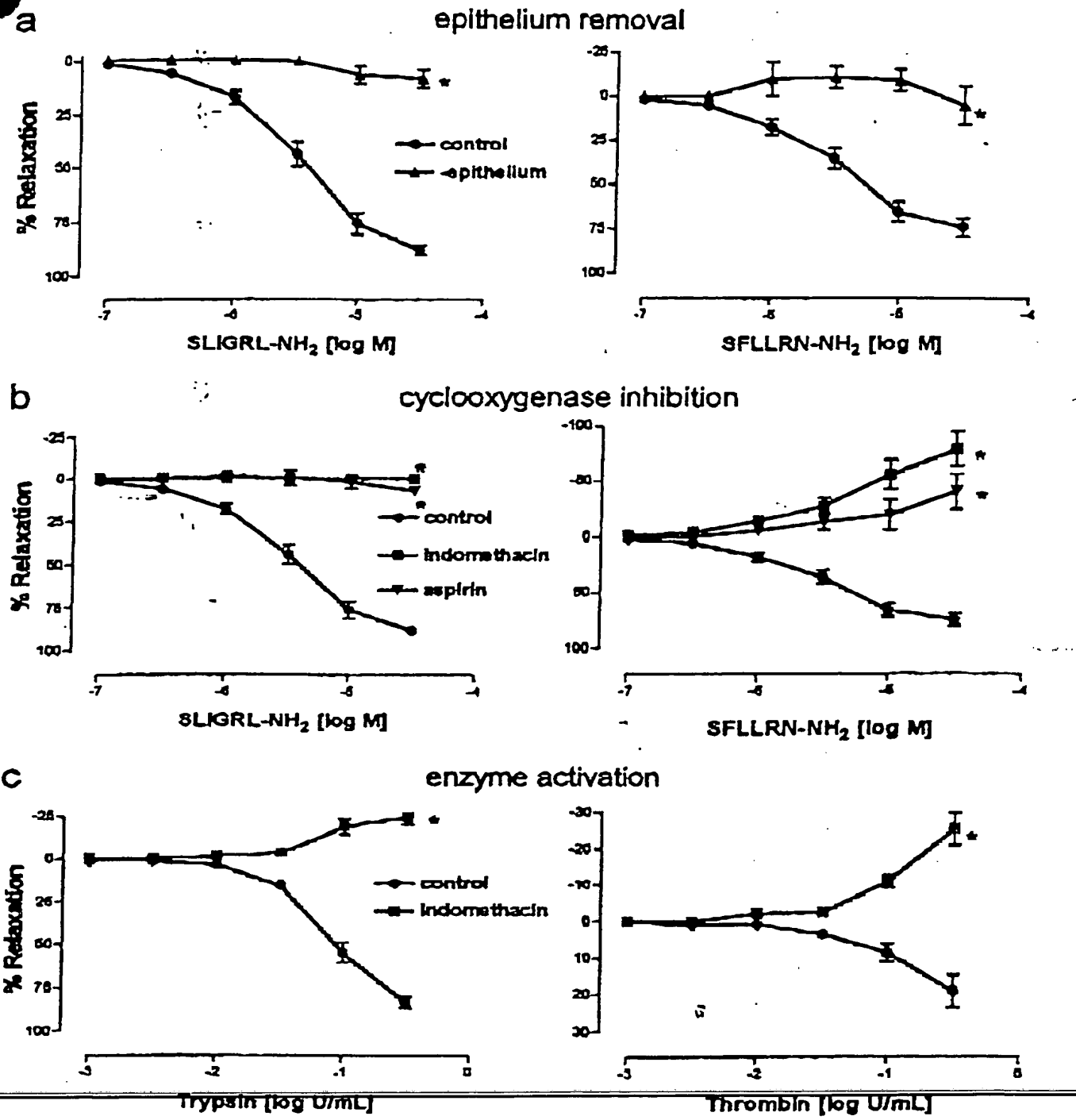

a
epithelium removal
% Relaxation
control
epithelium
SLIGRL-NH2 [log M]
SFLLRN-NH2 [log M]
b
cyclooxygenase inhibition
% Relaxation
control
indomethacin
aspirin
SLIGRL-NH2 [log M]
SFLLRN-NH2 [log M]
c
enzyme activation
% Relaxation
control
indomethacin
Trypsin [log U/mL]
Thrombin [log U/mL]

Figure 27

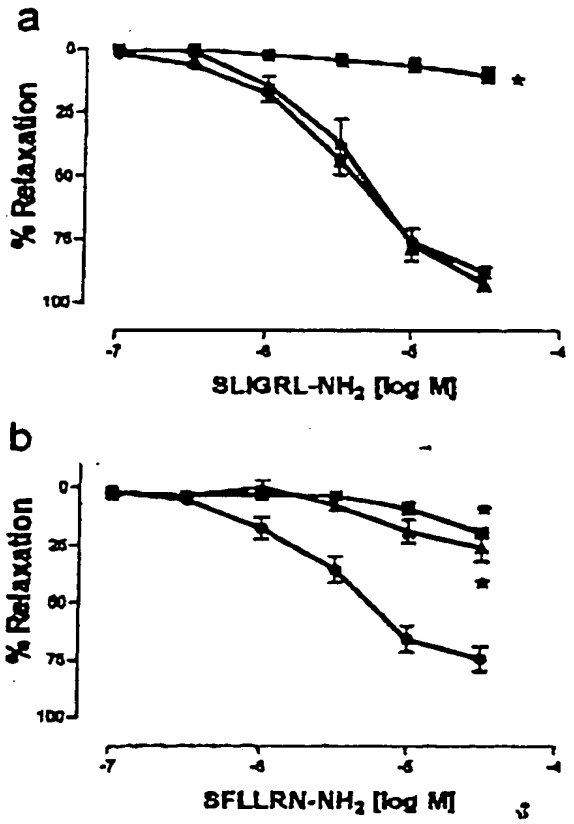

a
% Relaxation
0
25
50
75
100
-7
-6
-5
-4
SLIGRL-NH2 [log M]
b
% Relaxation
0
25
50
75
100
-7
-6
-5
-4
SFLLRN-NH2 [log M]

Figure 28